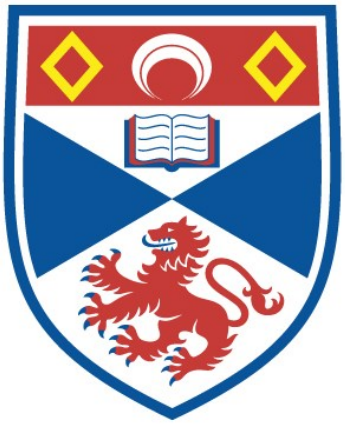
ANTIBACTERIAL ACTIVITY IN THE BLOOD CELLS OF 'CARCINAS MAENAS' (L.) AND OTHER CRUSTACEANS

June R. S. Chisholm

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Antibacterial Activity In The Blood Cells Of *Carcinus maenas* (L), And Other Crustaceans

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Submitted for the Degree of Doctor of Philosophy in the University of St. Andrews

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May, 1993

Dedication

For Derrick and Hilton, and in memory of Phyllis

Declaration

a) I, June Romayne Shirley Chisholm, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial / complete fulfillment of any other degree or qualification.

Signed Date 6th May 1993

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Abstract

In vitro antibacterial activity in the haemocytes of *Carcinus maenas* (L) was investigated. Haemocyte lysate supernatants (HLS) were assayed against twelve Gram-positive and Gram-negative marine bacteria, eight of which proved sensitive to the antibacterial factor or factors contained therein. Antibacterial activity was also found in HLS from four other marine crustaceans- *Glyptonotus antarcticus, Galathea strigosa, Nephrops norvegicus* and *Crangon crangon*. Activity in *C. maenas* HLS was independent of divalent cations, operated at high titre, was stable after treatment at 100 °C for 30 minutes and also after three months storage at -70 °C; it was present in the granular cells (which also contain the prophenoloxidase (proPO) activating system, a putative recognition mechanism in arthropods), but was absent from hyaline cells and plasma.

In vitro studies were carried out to determine whether a relationship exists between the proPO system and the antibacterial activity in the haemocytes of *C. maenas*. It was shown that phenoloxidase and an activating serine protease were not responsible for the observed antimicrobial effects, although the possibility that activity resides with some other component of the proPO system has not been excluded.

Seasonal variation in antibacterial activity, haemocyte counts and HLS protein concentrations was found, with conspicuous depression of antibacterial activity at two key points in the year. This occurred in conjunction with extremes of water temperature and it is proposed that the variation in antibacterial activity is due to underlying temperature effects on haemocyte counts and protein levels.

Low levels of lytic activity against *Micrococcus luteus* cell walls and low levels of bacterial agglutination were recorded, but the powerful antibacterial activity in HLS could not be attributed to these alone. Gel filtration with Sephadex G-100 revealed at least three antibacterial proteins of differing molecular size, with estimated MW of 72 kDa, 34 kDa and 4 kDa. The 72 kDa and 34 kDa proteins were bacteriostatic and the 4 kDa protein was bacteriolytic.

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Abbreviations

α_2–M	α_2 Macroglobulin
ANOVA	Analysis of variance
BSA	Bovine serum albumin
CAC	Cacodylate buffer
CS I	Complete *Carcinus* saline
CS II	Modified *Carcinus* saline
EDTA	Ethylenediaminetetraacetic acid
GLS	Granular cell lysate supernatant
GPC	Gel permeation chromatography
HEWL	Hen egg-white lysozyme
HLS	Haemocyte lysate supernatant
HyLS	Hyaline cell lysate supernatant
Ig	Immunoglobulin
LPS	Lipopolysaccharide
MAC	Marine anticoagulant
MHC	Major histocompatibility complex
MW	Molecular weight
MWL	Molecular weight limit
NADPH	Nicotinamide adenine dinucleotide hydride
PNA	p-Nitroaniline
proPO	Prophenoloxidase

PTU	Phenylthiourea
SI	Survival index
SOD	Superoxide dismutase

Chapter 1
General Introduction

1.1. Introduction

The class Crustacea comprises a morphologically diverse array of arthropod invertebrates which exploit an equally diverse array of habitats. Typical representatives include crabs, lobsters, shrimps, prawns, barnacles, woodlice and sand-hoppers. Only a few crustaceans (such as the tropical land hermit crab (*Coenobita* sp.), the coconut crab (*Birgus latro*) and woodlice) have adopted terrestrial or semi-terrestrial lifestyles, and of the enormous number of aquatic species the majority are marine: worldwide there are over 30,000 marine species, ranging in habitat from the supralittoral zone to the abyss.

The members of the class share the general attributes of the Arthropod group in possessing a chitinous, segmented exoskeleton with jointed appendages and an open vascular system, but a number of features distinguish them from other arthropods. Characteristically, they develop from free-living nauplia larvae and, in the adult form, possess biramous appendages (of which two pairs are antennae and at least three pairs have been modified as jaws), and gills for respiration. This latter feature is replaced with special organs for air-breathing in the terrestrial representatives of the class. Trunk specialization varies but frequently there is a carapace which covers all or part of the body. In addition, the exoskeleton of large marine crustaceans is calcified. More detailed information about crustacean taxonomy, anatomy and physiology can be found in Abele (1982) and Mantel (1983).

The marine environment is rich in potential pathogens, and for a crustacean to survive and fulfil its reproductive potential (a cycle of events involving a vulnerable planktonic larval stage which is usually followed by several moults - Hartnell & Gould, 1988) it needs effective mechanisms to eliminate any micro-organisms or parasites which may invade the haemocoel.

Recent scientific interest in the immune mechanisms of crustaceans has evolved primarily through the growing economic importance of these animals. Over the last quarter of a century the crustacean fisheries industry has undergone rapid expansion and, in addition, there has been a marked development of crustacean aquaculture to fulfill a variety of needs. Consequently, research activities have been stimulated in a number of areas related to crustacean biology, not the least of these being in the field of crustacean immunity.

Although the main thrust of this thesis is directed at one particular aspect of antibacterial activity in crustacean haemolymph, it would be erroneous to consider this phenomenon in isolation; antibacterial activity represents just a single component of a complex system of inter-related cellular and non-cellular events and compartmentalizaton of the elements of the crustacean immune system serves only to narrow our view of the whole field of operations. Thus, the principal aim of this introductory chapter is to provide a review of the numerous defence strategies which are employed by crustaceans when challenged by potential pathogens. This is preceded by a brief discussion of the economic and ecological significance of crustaceans, together with an account of some of the relevent crustacean diseases encountered in both the natural environment and in aquaculture conditions.

1.2. Economic and Ecological Significance of Crustaceans

Historically, crabs, lobsters, crayfish, prawns and shrimps have been a popular and abundant source of high quality protein for those living in maritime states or, alternatively, have been prized as a luxury addition to the diet for those more distant from the sea. Shrimps collectively represent one of the most economically valuable fishery products. Tropical and subtropical penaeid shrimps provide the largest

contribution, whilst caridean shrimps, which are more widely distributed in cooler temperate regions, tend to be less commercially exploited (see Neal & Maris 1985 for review).

Less familiar, but nonetheless important, are the fisheries that land krill, mantis shrimps, mysid shrimps, copepods and zooplankton. These resources have several applications, such as the extensive use of krill, shrimps and copepods as bait and also as fish food in aquaculture. Copepods additionally provide food supplements in the pet food industry, whilst zooplankton is used as a nutritious additive rather than for direct consumption (Omori, 1978). Krill, with its high protein value, is a potentially important source of protein for the many areas in the world where there is a serious shortage of this essential component of the diet (Nicol & de la Mare, 1993).

For the larger crustaceans (crabs, lobsters and crayfish) the yield and quality of the product depends to a large extent on the anatomy of the animal and degree of development of the musculature. Lobsters have status as a luxury food and represented 5.5% of the total world marine crustacean catch in 1981 (see Cobb & Wang, 1985 for review). *Homarus* and *Nephrops* genera are heavily fished in the North Atlantic, the palinurid genera of spiny lobsters have a wide distribution in coastal areas of the Pacific and Atlantic oceans, whilst crayfish are most abundant in freshwaters in temperate latitudes (Cobb & Wang, 1985). In North America they are the subject of intense fishing and in Europe *Astacus* fisheries have existed since the sixteenth century, although stocks are now somewhat depleted due to disease (Johnson, 1983; Alderman *et al.*, 1984).

Brachyurans and anomurans (true crabs and squat lobsters) are also widely distributed, with *Cancer magister* (Dungeness crab), *Callinectes sapidus* (blue crab) and *Chionoecetes* sp. (snow crab) dominating world landings for food supplies (see Haefner, 1985 for review). *Uca* sp. (fiddler crabs) and *Carcinus maenas* (shore

crab) are frequently used as bait although *C. maenas* is also used as food in parts of Europe (Haefner, 1985).

In an effort to meet the increased market demand for crustaceans, landings from natural resources (which in some cases have been exploited to the maximum) are now supplemented by animals reared in culture conditions. Crustacean culture has been in evidence since the early 19th century (see Provenzano, 1985a for review) but the last few decades has seen an increase in production, particularly with respect to shrimp farming. There have also been attempts to restock or supplement natural habitats with shrimps, crabs, lobsters and crayfish, which has required the large scale rearing of crustaceans. In this area the main success has been with crayfish (Provenzano, 1985a).

Crustaceans such as *Artemia* (brine shrimp) are reared intensively and used as a food source for other animals, particularly commercially raised fish. It is also a common practice to mass rear cladocerans in sewage or waste lagoons to improve the water condition (Norman *et al.*, 1979). Comprehensive reviews of the principles and commercial culture of crustaceans may be found in Provenzano (1985a,b).

There is, however, a negative side to the story. It would be difficult, for example, to quantify the effect that crustaceans such as barnacles may have on maritime maintenance costs and fuel consumption. Provenzano (1985c) has suggested that the annual cost probably lies somewhere in the region of hundreds of millions of dollars. Similarly, the damage that isopods inflict as they burrow into and feed on submerged timbers is well documented and substantial (Schmitt, 1973) and dates from the early 19th century when the first recorded identification of a wood boring isopod in Great Britain was made by Robert Stevenson in 1811 (cited in Calman, 1911).

Also, there are several species of parasitic crustaceans that affect fish stocks both in the natural environment and in aquaculture conditions. The Branchiura, Copepoda,

Isopoda, Amphipoda, Cirrepedia and Ostracoda all have representatives that cause disease, although of these the parasitic copepods are generally considered to have the widest range of interactions (Kabata 1984). Effects range from local tissue damage at the site of parasite activity, to general systemic effects which are life-threatening. The latter include weight loss, inadequate growth, reproductive failure, aberrant behaviour, blood abnormalities and secondary infections (Kabata, 1984). Diminished health of the stock and reduction in growth rates affects the total yield of the product and hence its commercial value. Nonetheless, according to Kabata (1984), it remains difficult to assess the overall impact of crustacean parasitic infections on the fisheries industry.

Crustacean parasites are not only found in association with fish; they have a wide range of hosts which include sponges, cnidarians, annelids, molluscs, echinoderms and other crustacea (Cressey, 1983). Nor are they exclusively aquatic; for example, the pentastomids (once considered to be an obscure and isolated phylum) are parasites of the respiratory tract of all classes of tetrapods and are now regarded by many as belonging to the class Crustacea (see for example, Riley *et al.*, 1978; Storch & Jamieson, 1992).

From an ecological perspective, crustaceans are an important part of the food web and occupy diverse ecological niches throughout the different stages of their life cycles. Microscopic zooplankton, which feeds on phytoplankton, bacteria or fungi, includes larval forms of crustacea and is of primary importance in the aquatic food chain. Krill constitutes food for whales whilst adult crustaceans generally perform scavenging or filter feeding operations and are themselves preyed upon by other aquatic organisms such as octopus, fish or sea otters. Crustaceans are overwhelmingly dominant in some habitats, such as reefs, water column and sandy beaches; an informative account of populations and communities of the Crustacea may be found in Coull & Bell (1983). It is clear therefore, even from this very abbreviated

account, that crustaceans are far from being insignificant members of the animal kingdom.

1.3. Diseases of Crustaceans

1.3.1. Economic implications and causal factors

The development of aquaculture has, in general, made crustacean food items more available and demand dictates that they be produced at reasonable prices. Thus, from an economic standpoint, it has become necessary to optimize the culture strategies and control disease within the stock.

Most, but not all, crustacean culture is carried out in semi-intensive monoculture systems where there is a certain amount of water recycling and some modificaton of water quality during capture time. Kinne (1976) provides an excellent review of marine culture systems and Provenzano (1985a,b) describes in detail the practices employed for the major commercial decapod systems.

As with any system of monoculture, one of the major problems facing the aquaculturist is prevention and control of disease; failure to address this adequately can result in heavy financial losses for the industry. The very nature of monoculture encourages and exacerbates the development and transmission of disease, which tends to be rapid - much more so than in polyculture systems where a level of natural control frequently operates to limit the spread of infective agents. An immediately obvious parallel exists in our present-day agricultural practices .

The routine holding of concentrated numbers of individuals in stressful environments (such as shedding tanks) predisposes the animals to diseases which may result in epidemics with associated high mortalities. Under such artificial

conditions there may be physical and chemical changes in water quality due to accumulation of metabolites, production of ectocrines and increase in water turbidity. This in turn may lead to depressed oxygen levels, elevated pH and reduced growth rates in the stock (Provenzano, 1985a). In this context it is interesting to note that for *Macrobrachium* sp. (tropical caridean shrimps, commercially cultivated in many parts of Northern and Southern America), most reports of viral and microbial infection are considered to be opportunistic and related to poor husbandry rather than to the occurrence of obligate pathogens for the species (Sindermann & Lightner, 1988).

Concurrent with the growth of the crustacean aquaculture industry has been the identification and continued study of numerous associated diseases. This is not an appropriate place for a treatise on the aetiology and treatment of shellfish disease, and there are a number of excellent texts which cover the subject in depth, for example Sindermann & Lightner (1988). However, there are certain pathologies of major importance which merit discussion and are dealt with in sections 1.3.2-1.3.5. The bulk of economically significant Crustacean diseases are almost exclusively viral and microbial in origin, with the latter category embracing rickettsiae, bacteria, fungi and protozoa. Helminths and parasitic crustaceans are also known to have an impact on shellfish populations (Sindermann & Rosenfeld, 1967; Sindermann, 1968) and are difficult to control because of their frequently complex life cycles. Since this thesis concerns antibacterial activity in crustacean haemolymph, discussion will be limited to some of the more serious or familiar microbial diseases that affect this group.

1.3.2. Protozoan diseases

The first protozoan disease of economic interest, occurring in soft-shell and moulting blue crabs (*Callinectes sapidus*), was described by Sprague and Beckett in 1966, although the true nature of the disease did not become apparant until 1968 when *Paramoeba* species was identified as the causal agent (see review by Johnson,

1977). Subsequently, the rock crab (*Cancer irroratus*) and the lobster (*Homarus americanus*) have been described as additional hosts (Sawyer, 1976). Several other parasitic protozoans, including dinoflagellates, gregarines, cnidosporans and ciliates, are reported in the literature and have been implicated in unexplained mortalities in natural and laboratory reared stocks (Vey & Vago, 1973; Sawyer & MacLean, 1978). Many of the ciliates cause fouling diseases which affect the gills and appendages and result in hypoxia, whilst other protozoans are found as endoparasites.

Anophrys sarcophaga is an example of an endoparasitic holotrichous ciliate which was first described for *Carcinus maenas* by Poisson in 1930 (cited in Couch, 1983) and has since been found in *Cancer* species as well (Bang, 1983). *Anophrys* ingests the haemocytes of the host animal and eventually enormous numbers of ciliates are present in the haemolymph, which takes on a milky appearance. Secondary bacterial infection usually ensues and *Anophrys* then feeds on the bacteria in the moribund host (see Sparks *et al.*, 1982; also review by Bang, 1983). Descriptions of other commercially important protozoan infestations of crustacea can be found in Couch (1983).

1.3.3. Rickettsial diseases

The rickettsiae are a group of extremely small Gram-negative organisms (of the order of 0.2-0.7 x 0.8-1.6 μm) which are similar to bacteria. Frequently associated with insects and arachnids, they have also been reported in terrestrial and aquatic crustaceans, including isopods, marine crabs and penaeid shrimps (for example, Anderson *et al.*, 1987; also review by Johnson, 1983). The penaeid shrimp infection targets the hepatopancreas (Sindermann, 1986), particularly in juvenile shrimps, and has resulted in high mortality in experimental situations as well as in cage and pond-cultured animals in Hawaii and Malaysia. A disease syndrome, which implicates rickettsia as the primary causative organism, has been described for *Penaeus*

monodon and in this animal rickettsial lesions have been found in heart, hepatopancreas, gills and parietal haemocoel linings. In the crab *Carcinus mediterranus*, rickettsial infection occupies the connective tissues (Bonami & Pappalardo, 1980) and experimental infections cause death in 15 days.

1.3.4. Fungal diseases

Fungal disease has been reported in all commercially important species of crustacean and useful reviews can be found in Unestam (1981), Johnson (1983) and Smith & Söderhäll (1986a). Probably the most notorious disease is the so-called freshwater crayfish plague, caused by the oomycete *Aphanomyces astaci*. This fungus has been responsible for the virtual elimination of the indiginous European species *Astacus astacus* and has been implicated in sporadic reports of severe crayfish mortalities in the United Kingdom (Alderman *et al.*, 1984). It is thought that the crayfish plague was introduced from North America where another species of crayfish, *Pacifastacus leniusculus*, is highly resistant to this infection and has become a reservoir for the disease in Europe (Unestam, 1975). *Aphanomyces astaci* gains access through damaged areas of exoskeleton or by means of extracellular chitinases and proteases (Nyhlén & Unestam, 1975, 1980), resulting in an invasion of the endocuticle with fungal hyphae which develop into a dense mycelium (Nyhlén & Unestam, 1975, 1980). Ultimately there is involvement of muscle and internal organs, resulting in weakness and death. A comparable fungal infection, black mat disease, has been reported in the tanner crab, *Chionoecetes bairdi* and the causal agent identified as an ascomycete, *Trichomaris invadens* (see Sparks & Hibbits, 1979 ; Hibbits *et al.*, 1981). Studies carried out by Unestam (1975) indicate that species of crayfish from Australia and New Guinea are also susceptible to the disease and it has been proposed that should *A. astaci* be introduced to that part of the world, the consequences would be disastrous.

In other crustaceans, fungal diseases such as *Leptolegnia, Lagenidium* and *Fusarium* sp. are known to affect ova and embryonic stages (for examples see, *C. sapidus:* Sandoz *et al.*, 1944; *Cancer magister:* Armstrong *et al.*, 1976; *H. americanus:* Nilson *et al.*, 1976), as well as post-larval stages (*H. americanus:* Lightner & Fontaine, 1975; *Macrobrachium rosenbergii:* Burns *et al.*, 1979). Fungal infections occur in both wild and cultured populations of crustaceans, although infections of eggs and larvae tend to be less common in the wild (Johnson, 1983).

1.3.5. Bacterial diseases

A multitude of crustacean diseases have been ascribed a bacterial aetiology, the most notable of which is Gaffkaemia, a septicaemia which is otherwise known as the Red-Tail Disease of lobsters. High market demand for lobsters, especially in the United States of America, has resulted in an economic incentive to understand and control this highly transmissible infection.

The majority of marine bacteria are Gram-negative but Gaffkaemia is unusual in that the causative agent is a Gram-positive coccus, *Aerococcus viridans* (var.) *homari* (previously, and still commonly, referred to as *Gaffkya homari*) (Hitchner & Snieszko, 1947). Few early symptoms of disease are noticeable but eventually heavily infected lobsters become lethargic and "lie quietly with their chelipeds extended" (Rabin, 1965). Diagnostic features are a prolonged haemolymph clotting time and the identification of *Aerococcus* tetrads in blood smears (Rabin, 1965; Stewart,1975; Johnson *et al.*, 1981). Infection arises via an injury of some kind and fatality can result from an inoculum as small as 10 organisms per Kg bodyweight (Cornick & Stewart, 1968a; Stewart & Rabin, 1970), and although antibiotic treatment has been successful in a limited way, this is heavily dependant on an early diagnosis of the disease (Stewart & Arie, 1974). Prophylactic measures include hypochlorite treatment of holding areas, and a degree of resistance can be conferred

on the animals by the use of attenuated organisms or formalin-killed pathogens as vaccines (Stewart & Zwicker, 1974). An associated cause for concern is the evidence that crabs and other crustaceans may act as reservoirs for the disease; experimental infections have been induced, for example, in *C. irroratus* (Cornick & Stewart, 1968b), in the spiny lobster *Panulirus interruptus* (Schapiro *et al.*, 1974), as well as in several other marine species (see review by Johnson, 1983).

Another less obviously harmful condition is shell disease, caused by chitinolytic bacteria and commonly affecting lobsters, crabs, shrimps and other crustaceans. The disease was first described for lobsters by Hess (1937) and since then the genera *Vibrio, Beneckea, Pseudomonas* and *Spirillum* have been variously implicated (see Sindermann & Lightner, 1988; Cipriani *et al.*, 1980) as the commonest causal agents. Although shell disease is not fatal *per se,* the resultant necrotic lesions encourage secondary infections which may be rather more serious. Also, the appearance of the animal is blemished, making it unacceptable as a marketable item. Shell disease presents initially as brown marks on the exoskeleton which eventually coalesce into irregular areas with depressed necrotic centres. Baross *et al.* (1978) consider that two processes operate in concert, with an initial slow microbial degradation of the epicuticle which allows the subsequent penetration of chitinolytic bacteria. Generally, the diseased exoskeleton is lost during ecdysis unless there has been secondary involvement of the underlying soft tissues. For further details, an interesting and informative account of shell disease can be found in Johnson (1983).

Several Gram-negative pathogens have been isolated from the blue crab *C. sapidus,* and halophilic species of *Vibrio* and *Pseudomonas* have been described as the cause of acute and chronic haemocoelic bacterial infections in crabs, lobsters and shrimps (see Johnson, 1983 for review). Reports of this disease are mainly associated with artificially enclosed crustaceans although Vey *et al.* (1975) have also described it in stressed natural populations. The chronic infection is characterized by

an increased intermoult time and a failure to complete the moult successfully, whereas the acute condition is usually fatal within six days (McKay & Jenkin, 1969). A feature of both forms of the disease is an increased haemolymph clotting time and a reduction in the number of circulating haemocytes. This could well be an opportunistic infection since the organisms involved are naturally present in the surrounding water and sediments, and reports of the disease are nearly always related to unfavourable environmental conditions for the host. Additional information is given in Johnson (1983), who provides a comprehensive and stimulating overview of viral, rickettsial, bacterial and fungal diseases in a wide range of crustaceans.

The above repertoire of crustacean diseases is far from exhaustive and only serves to highlight some of the problems faced by the aquaculture industry. A particularly interesting characteristic, common to many microbial infections in crustaceans, is the involvement of the haemolymph, frequently with effects on clotting and haemocyte counts. Crustacean haemocytes have a number of important functions, including transport, synthesis, storage, and wound repair (Ratcliffe & Rowley, 1979). They also play a significant role in host defence so, in common with the vertebrate system, disease that directly or indirectly compromises the immune system may render the animal susceptible to lethal superinfections.

It can be seen that for the aquaculturist to successfully convert low-cost foodstuffs into high protein shellfish and market it at competitive prices, a scientific approach to the prevention and control of disease is of paramount importance. An integral part of this approach must be an understanding of the immune systems of the animals involved.

1.4. Immune Defence Systems of Crustaceans

Immunology, as a scientific discipline, has its roots in the first observations of phagocytosis in an invertebrate, *Daphnia*, reported by Metchnikoff in 1884 (cited in Ratcliffe *et al.*, 1982). As a direct consequence of this, fundamental questions were raised concerning the nature of non-self recognition and from this evolved the concept of cellular and humoral immunity. These terms, 'humoral' and 'cellular', were useful in the early years of immunological research (which inevitably tended to concentrate on mammalian systems) and a vast body of literature now exists in relation to vertebrate immunity, largely prompted by the need to control human disease processes. Yet it is debatable whether this terminology is appropriate for invertebrates which thus far seem to lack the array of immunologically competent molecules (such as immunoglobulins) which form the humoral components of the immune system in vertebrates.

In contrast to the vertebrate system there is a relatively poor understanding of invertebrate immune mechanisms. Of the arthropod group it is the insects, crustaceans and chelicerates which have attracted particular research interest because of their economic or biomedical applications. Aside from the crustaceans, which have already been discussed, it is demonstrably clear that insects (whilst having many benefits to offer) contribute in a major way to pest problems and are vectors of numerous agricultural, animal and human diseases. Chelicerates, particularly the horseshoe crab *Limulus polyphemus*, have made an enormous contribution in the field of medicine with the development of an assay that utilises particular properties of the *Limulus* amoebocytes to detect endotoxins in intravenous solutions (Levin & Bang, 1968).

For crustaceans, protection from infection operates at a number of different levels, starting with the substantial physical barrier of an exoskeleton which presents a hard and relatively impenetrable outer layer. However, this barrier may be breached during

ecdysis or following wounding and, if the physical integrity of the host is damaged, the more fragile internal milieu becomes exposed to obligate or opportunistic pathogens. Once this has occurred, a multiplicity of non-specific responses are brought into play in an attempt to contain or eliminate infection.

Haemolymph clotting, phagocytosis, agglutination, precipitation, antibacterial, antifungal, antiviral and cytotoxic factors represent the broad categories of strategies employed and in addition there is a repertoire of cytokine-like molecules, opsonins and cell adhesion molecules which augment the cellular defences (Table 1.1). Many responses are allied to haemocyte activity although in a small number of cases the factors appear to reside exclusively in the plasma and operate independently of ciculating haemocytes (see recent reviews by Söderhäll & Cerenius, 1992; Smith and Chisholm, 1992). All these responses (with the possible exception of the clotting response to injury) are consequent upon the discrimination between self and non-self molecules, which brings us back to the key question of recognition raised by Metchnikoff's observations in the last century.

1.4.1. Recognition

For invertebrates, the fundamental question about recognition of self and non-self has yet to be satisfactorily answered and, as suggested by Cooper *et al.* (1992), probably represents the area of immune response where vertebrates and invertebrates show the greatest differences. Lackie (1986) has presented two alternative hypotheses that could be addressed with respect to recognition. One postulates that intact self is ignored but non-self is actively recognized and stimulates a response, whereas the other, conversely, postulates that intact self requires active recognition and failure of recognition triggers a response.

From our understanding of mammalian recognition systems it would appear that for vertebrates the answer lies somewhere between the two, with active recognition of

Table 1.1

Table 1.1. Host defence strategies in crustaceans and other invertebrates

Strategy	Function	Crustaceans	Other Invertebrates
Agglutination	Aggregates foreign particles. Includes bacterial agglutinins, haemagglutinins and/or lectins.	Present in nearly all species. Appear to aid sequestration of infective agents. Little evidence for a role in recognition for lectins.	Widely distributed in all groups. Some evidence that lectins mediate in recognition in molluscs & insects.
Killing Factors:			
Lytic agents	Bactericidal.	Bacteriolysins & lysozyme seldom reported. Haemolysins present in spiny lobsters.	Bacteriolysins & lysozyme present in most groups, especially molluscs & insects.
Peptides & other bioactive molecules	Bactericidal.	Not known.	Cecropins and attacins in insects.
Pigments	Microbicidal.	Melanin or its precursors are fungicidal & possibly bactericidal.	Melanin & its precursors in insects. Echinochrome A in echinoderms.
Neutralizing factors	Anti-viral.	Reported for *Callinectes sapidus* & *Carcinus maenas*.	Occasional reports but poorly understood.
Cytotoxic agents	Destroy cells.	Crayfish granular cells are cytotoxic for normal & tumour vertebrate cells *in vitro*.	Demonstrated for various phyla. Mechanism unknown.
Precipitins	Sequester soluble 'antigens' from blood.	Few reports (mostly in older literature).	Few reports.

Cytokines	Non-antibody proteins with diverse immunological & homeostatic functions. Produced by blood cells.	Few reports. Factors associated with proPO[1] activation influence exocytosis, phagocytosis & cell adhesion in decapods.	Poorly understood. Some reports for echinoderms.
Modulators	Regulate the activities of immunologically aggressive molecules.	Poorly studied. α_2 Macroglobulin cages proteinases & is found in crayfish plasma.	Poorly studied. α_2 Macroglobulin found in insect & chelicerate blood.
Clotting factors	Prevent blood loss & seal wounds.	All species. Involves plasma gelation as well as cell aggregation.	Cell aggregation in all coelomate groups. Only arthropods display plasma gelation as well as cell aggregation.
Recognition factors	Bind specifically to non-self molecules & trigger cell responses.	proPO[1] factors released from cells. β 1,3-glucan-binding factor found in crayfish plasma.	proPO[1] factors and glucan-binding factor present in some arthropods. Some evidence for lectin-mediated recognition in insects and molluscs.
Phagocytosis	Removal of non-self particles or damaged /degenerating cells. Non-specific response.	Associated primarily with hyaline cells. Observed levels generally low *in vitro* even in presence of opsonins.	Associated primarily with plasmatocytes (insects) or phagocytic amoebocytes (other inverts).
Encapsulation	Sequestration of non-self particles in multilayered sheaths of flattened haemocytes. Response to particles that are too large to be phagocytosed.	Widely reported. Melanization of capsule occurs due to degranulation of haemocytes and activation of proPO system.	Reported for most invertebrate phyla. Melanization of capsule in insects due to proPO[1] activation followng degranulatiion of haemocytes.
Nodule formation	Immobilisation of large numbers of microbes by clumping following exocytosis of granular cells.	Considered to be a hybrid of the phagocytic and encapsulation reactions. Heavily melanized.	Recorded for insects. Clumps composed almost entirely of granular cells. Attachment of plasmatocytes occurs later.

[1]proPO: prophenoloxidase activating system.

non-self being predicated on the ability to also recognize self. In vertebrates, members of the immunoglobulin (Ig) gene superfamily have been identified as playing a critical role in generating a vast range of specific receptors for antigen recognition (see review by Turner, 1992). Other members of the family, such as major histocompatibility complex (MHC) molecules (crucial for the discrimination between self and non-self), are inolved in antigen non-specific immune regulation (Turner 1992).

Unfortunately, no such certainties exist for the invertebrates as a whole, although there is some evidence (based on serological studies and partial amino acid sequence analyses) that C-reactive proteins and molecules of the Ig superfamily do exist in these animals (see reviews by Marchalonis & Schluter, 1990 and Cooper *et al.*, 1992). Considerable research into histocompatibility has been carried out for several invertebrate phyla (for examples see Bigger *et al.*, 1981 (sponges); Theodor, 1970 and Ivker, 1972 (cnidarians); Cooper, 1965 and Cooper & Roch, 1986 (annelids); Coffaro & Hinegardner, 1977 (echinoderms); Raftos *et al.*, 1987 and Raftos, 1988 (tunicates)) and in these animals studies have focussed on responses to allogeneic and xenogeneic grafts (see also review by Hildemann, 1979).

It has been suggested that MHC molecules did not arise as a novelty for vertebrate immune recognition (Weissman, 1988) and a good case can be argued for the evolution of the MHC system in invertebrates, particularly if one considers the responses found in marine colonial or encrusting forms. Weissman (1988) has proposed that originally the MHC may have been a functionally adaptive strategy, a theory that gains credence when the evidence from sponges, cnidarians and tunicates is considered. Tunicates, many of which are colonial in habit, are primitive chordates and occupy a key phylogenetic position between invertebrates and vertebrates. It has been found that a special set of alleles play a role in recognition whereby juxtaposed colonies sharing just one common recognition allele at a single, highly polymorphic locus, will fuse and form a chimaera (see Weissmann *et al.*, 1990 for review). In the

absence of any common recognition allele at the recognition locus, a necrotic zone develops between the two colonies, even if they have other genetic similarities. In some instances (where there are species differences) one colony will completely overgrow the other; a similar sequence of events has been observed in encrusting sponges and cnidarians (Cooper *et al.*, 1992), and may be regarded as a strategy whereby the integrity of adjacent colonies is maintained.

Although Scofield *et al.* (1982) have shown that protochordate recognition is controlled by an MHC-like gene system, in general, the genetic basis for invertebrate recognition is still largely unsolved. Thus far there has been no identification of recognition loci in arthropods but, for insects and crustaceans at least, there are two important elements of the arthropod immune system that emerge as possible agents of recognition. One putative mechanism is the prophenoloxidase (proPO) activating system (see section 1.4.2) which occurs primarily in insects and crustaceans; the other possibility is that cell-bound lectin molecules may function in recognition (see section 1.4.5). Herein lies the fundamental difference between vertebrate and invertebrate systems: vertebrates and many invertebrates have lectin receptors which bind to LPS and other microbial carbohydrates (Greenberg, 1987, cited in Cooper *et al.*, 1992) but only vertebrates have evolved the specific receptors and associated rearranging genes that permit recognition of highly specific molecules (Cooper *et al.*, 1992).

Early work in comparative immunology tended to concentrate on efforts to identify adaptive immune responses in lower animals; by extrapolation it could then be postulated that the presence of adaptive responses would indicate the presence of molecules analogous to the immunoglobulins of vertebrates (see Burnet, 1968, 1971) However, as a consequence of considerable research effort, it is now generally accepted that invertebrates lack specific immunoglobulins and cannot mount anticipatory immune reactions (Klein, 1989). Indeed, according to Klein (1989), it

could be argued that in the presence of an open circulatory system it is probably more appropriate that the host response should be rapid and non-inducible, thus avoiding an overwhelming invasion of the haemocoel by potential pathogens.

1.4.2. The prophenoloxidase activating system

The prophenoloxidase (proPO) activating system is a complex cascade of serine proteases and other factors which reside in the granular and semigranular cells of arthropod haemolymph (Johansson & Söderhäll, 1985). In insects it appears that this cascade system is also present in the plasma, whereas in crustaceans it is restricted to the haemocytes, from whence it is released by exocytosis. A substantial number of papers have been published in relation to the proPO system (for example see reviews by Ratcliffe *et al.*, 1985; Smith & Söderhäll, 1986b; Söderhäll & Smith, 1986a,b; Söderhäll, 1992) and to date the research has mainly centred on insects and crustaceans, although recent work by Jackson *et al.*, (1993) has indicated the presence of elements of this cascade in the solitary tunicate *Ciona intestinalis*.

The terminal component of the proPO cascade is prophenoloxidase, recently purified from crayfish haemolymph and shown to have a molecular mass of 76 kDa (Aspan & Söderhäll, 1991). In this animal the proenzyme is converted to the active form phenoloxidase by a serine protease with a molecular mass of 36 kDa (Aspan *et al.*, 1990). Phenoloxidase itself is a redox enzyme with a molecular mass of 60 kDa (Aspan & Söderhäll, 1991) which oxidises phenols to quinones. These purifications have also been carried out in insects and are reviewed in Söderhäll (1992).

The *in vitro* activity of phenoloxidase can be measured spectrophotometrically in haemocyte lysate supernatants (HLS) using L-dopa as the substrate (Söderhäll & Unestam, 1979) and the resultant quinones are capable of spontaneously forming melanin as an end product. This reaction is essential for the melanization of the exoskeleton of arthropods (Götz & Boman, 1984), following ecdysis or wounding,

and melanized areas are frequently seen at sites of injury. It is known that at ecdysis large numbers of cells which carry the cascade (granular and semi-granular cells) disappear from the circulating haemolymph, presumably to fulfil their role in restoring the protective exoskeleton (Vacca & Fingermann, 1983).

The proPO cascade is also considered to play an important role in crustacean immunity. Early work by Söderhäll & Unestam (1979) showed that fungal ß-1,3 glucans induce activation of crayfish prophenoloxidase. This is a highly specific response for this group of carbohydrates (Söderhäll & Unestam, 1979) and has been confirmed subsequently in a number of arthropod species (see reviews by Söderhäll, 1982; Ratcliffe *et al.*, 1985; Söderhäll & Smith, 1986b). Other microbial carbohydrates (lipopolysaccharides (LPS) and peptidoglycans) also elicit this response in a similarly specific manner (Söderhäll & Häll, 1984; Ashida *et al.*, 1974).

To date, a number of proteins of the proPO system, together with associated factors, have been isolated in both crustaceans and insects (see review by Söderhäll, 1992). Apart from the aforementioned phenoloxidase, prophenoloxidase and the activating serine protease, studies of crayfish plasma have revealed the presence of a serine protease inhibitor (Hergenhahn *et al.*, 1987), an α_2 macroglobulin, (Hergenhahn *et al.*, 1988; Stöcker *et al.*, 1991), and a ß-1,3 glucan binding protein (Duvic & Söderhäll, 1990) which induces spreading and degranulation of the granular haemocytes when reacted with a ß-1,3 glucan (Barracco *et al.*, 1991).

Liang *et al.* (1992) have recently demonstrated that the α_2 macroglobulin, although originally identified in plasma, is in fact synthesised by the haemocytes. The ß-1,3 glucan-binding-protein appears to be present in the circulating haemocytes as well as the plasma (Duvic & Söderhäll, 1990), but whether it is secreted by these cells is, at present, unknown. Also, a ß-1,3 glucan-binding-protein membrane receptor from crayfish blood cells has been purified and partially characterized (Duvic

& Söderhäll, 1992). Additionally, in the crayfish, there is a 76 kDa cell adhesion, degranulating and encapsulation promoting factor (Johansson & Söderhäll, 1988, 1989; Kobayashi *et al.*, 1990) which is synthesised by the haemocytes and resides in inactive form in the secretory granules of semigranular and granular cells (Liang *et al.*, 1992). The isolation of these molecules has lead to the proposal that the cascade is part of the recognition and defence mechanism in crustaceans, giving a rapid but controlled response to the presence of non-self molecules. The argument for this was set out initially by Söderhäll (1982) on the strength of of the fact that prophenoloxidase could be elicited to its active form by minute amounts of microbial polysaccharides (Unestam & Söderhäll, 1977; Söderhäll & Häll, 1984) and has gained credence in the light of recent research (see recent review by Söderhäll, 1992). Since the process of recognition is such a fundamental issue, it is worth examining the central features of the cellular responses as they relate to the proPO cascade in crustaceans.

Density gradient cell separation has been used to partition the three main haemocyte populations and has shown that semigranular cells contain only small amounts of prophenoloxidase (Söderhäll & Smith, 1983), are extremely labile and readily degranulate in the presence of non-self stimuli (Söderhäll *et al.*, 1986). They are especially sensitive to the elicitors of the proPO system cited above, namely microbial polysaccharides (Söderhäll & Smith 1983; Smith & Söderhäll, 1983a; Johansson & Söderhäll, 1985; Söderhäll *et al.*, 1986), whereas granular cells, by contrast, contain large amounts of prophenoloxidase (Söderhäll & Smith, 1983) but do not readily degranulate in the presence of microbial polysaccharides unless active proPO proteins are also present (Smith & Söderhäll, 1983b; Johansson & Söderhäll, 1985; Söderhäll *et al.*, 1986). Hyaline cells, the main phagocytic haemocytes in crustaceans, lack phenoloxidase (Söderhäll & Smith, 1983) but their phagocytic capability is enhanced *in vitro* by the presence of components of the proPO cascade

(Söderhäll *et al.*, 1986) which appear to function opsonically. Thus the sequential degranulation of semigranular and granular cells, modulated and controlled by the associated factors cited above, would appear to result in the release of signals to the phagocytic cells that permit recognition of non-self molecules. As well as the possible role for the proPO system in recognition and opsonin production, the end product of prophenoloxidase activation (melanin), and some quinones in the melanin pathway, have been found to have antifungal and antibacterial properties (Kuo & Alexander, 1967; Söderhäll & Ajaxon, 1982; St. Leger *et al.*, 1988).

1.4.3. Phagocytosis and opsonization

Following Metchnikoff's observations of phagocytosis in *Daphnia* in 1884 (cited in Ratcliffe *et al.*, 1982) it became apparent that phagocytosis was a universal phenomenon and could be identified across the whole spectrum of the Animal Kingdom. Phagocytic cells serve to eliminate unwanted particulate material from the host. The particles may be self or non-self in origin (for example, damaged or decaying tissues, or microbes respectively), in which case phagocytosis forms part of the host defence system; alternatively phagocytosis may be a feature of normal metabolic processes as is seen, for example, in the feeding activities of lowly protozoans and, at the other end of the spectrum, in the remodelling activities of osteoclasts in vertebrate bone metabolism. Castle (1984) gives an interesting account of the biochemistry and role of phagocytosis in the context of the mammalian immune system.

In invertebrates phagocytosis is carried out by specific blood cell populations and is one of the non-specific responses in the armoury of the immune system. It has been reported variously in insects, annelids, molluscs, crustaceans, echinoderms and tunicates and useful reviews may be found in Bauchau (1981), Ratcliffe & Rowley

(1979), Ratcliffe *et al.* (1982) and Ratcliffe *et al.* (1985) and, more recently, Bayne (1990).

Phagocytosis in crustaceans has been documented for a number of species such as crabs (Smith & Ratcliffe, 1978; Johnson 1976, 1977 and review 1987), crayfish (Reade, 1968; McKay & Jenkin, 1970a,b; Tyson & Jenkin 1974a), shrimp (Fontaine & Lightner, 1974) and lobster (Cornick & Stewart, 1968a; Rabin, 1970; Paterson & Stewart, 1974; Paterson *et al.*, 1976). In common with other arthropods, crustaceans possess an open circulation with a haemocoel, which gives rise to the term haemocyte for the associated blood cells. According to Bauchau (1981), all three crustacean haemocyte types (hyaline, semigranular and granular) have phagocytic capabilities, but the least active of these are the granular cells. Unfortunately the literature abounds with synonyms for the various haemocytes, which can be confusing, but Bauchau (1981) provides a useful review which clarifies the terminology.

The sequence of events in phagocytosis is firstly recognition (which induces a chemotactic response - see reviews by Castle, 1984, Ratcliffe *et al.*, 1982 and Ratcliffe *et al.*, 1985), then ingestion and finally clearance of the foreign particle. According to Gupta (1986), the effectiveness of phagocytosis in invertebrates depends on the number of phagocytosing cells, the nature of the non-self material and the frequency of attack. Not all microorganisms are equally affected by phagocytes - for example, the lobster pathogen *Aerococcus viridans* (var.) *homari* (*Gaffkya homari*) is phagocytosed by lobster haemocytes but not destroyed (Cornick & Stewart, 1968a,b) and it is known that some species of viruses, rickettsiae, bacteria and protozoa can survive in the haemocoel without being phagocytosed at all (Bauchau, 1981). Levels of phagocytosis (i.e. the percentage of haemocytes showing a phagocytic response) have been recorded *in vitro* for a few crustaceans (for example, McKay & Jenkin,1970a; Paterson & Stewart,1974; Smith & Ratcliffe, 1978) and are low (2 % -15 %) when compared with levels in other invertebrates (for

example, 40 % in *C. intestinalis* and 60 % in *Mytilus edulis* (Smith & Peddie, 1992). However, these levels are not necessarily a reflection of the *in vivo* condition and even low levels of phagocytosis may be effective if a large number of phagocytic cells are present. Also, as shown for example by Smith & Ratcliffe (1978) for the shore crab, a single phagocyte can ingest a number of particles which serves to amplify the efficiency of the mechanism.

Phagocytosis is enhanced by opsonins - host molecules which attach to the non-self particle and make it more recognizable to the phagocyte - and for invertebrates there have been several studies which demonstrate the presence of opsonic factors in the haemolymph (see Ratcliffe *et al.*, 1985 for review). Nevertheless, the true nature of invertebrate opsonins has yet to be fully resolved and there are conflicting reports with repect to the identity of these molecules. Some workers have presented evidence for serum agglutinins being the opsonin (for examples, McKay & Jenkin, 1970a; Tyson & Jenkin, 1974a; Van der Knaap *et al.*, 1982; Yang & Yoshino, 1990; Richards & Renwrantz, 1991; also review by Renwrantz, 1983), whilst the contrary view that they are distinct molecules finds some support in the work of Stuart (1968), Scott (1971) and, more recently, Goldenberg & Greenberg (1983). In a review of arthropod cellular immunity, Söderhäll & Smith (1986a) have discussed the apparent opsonic effect recorded by McKay & Jenkin (1970a) and Tyson & Jenkin (1974a) in the light of the recent findings on prophenoloxidase activation. They suggest that the observed increase in phagocytosis, noted after serum pre-treatment of the test particles, could be accounted for by induction of prophenoloxidase activating enzymes in the serum by specific microbial polysaccharides rather than by agglutinin activity.

There is a general dearth of information for invertebrates when it comes to the actual process of phagocytosis itself and the intracellular events thereafter; the majority of studies relate to insects, molluscs and annelids. In these animals there is

evidence that chemotaxis mediates in cell-foreign body contact (for example, Cheng & Howland, 1979; Howland & Cheng, 1982; Marks *et al.*, 1979; also review by Ratcliffe *et al.*, 1985) and, in molluscs, is related to the presence of small chemo-attractant molecules (1000 Da) on the cell walls of bacteria (Howland & Cheng, 1982). Also, it has been demonstrated in molluscs and insects that lysosomal enzymes include lysozyme, acid phosphatase, alkaline phosphatase, ß glucuronidase, amylase and lipase (see reviews by Ratcliffe *et al.*, 1982; Cheng, 1983; Ratcliffe *et al.*, 1985).

Rather less is known about intracellular events in crustacean phagocytes, although some lysosomal enzymes such as acid phosphatase (in *Homarus americanus* and *Carcinus maenas*) and leucine aminopeptidase (in *Oronectes virilis*) have been identified by Hearing (1969), White & Ratcliffe (1981) and Wood & Visentin (1967) respectively. In addition, ultrastructural studies of crayfish haemocytes have revealed the apparent fusing of granules and phagosomes (Sternshein & Burton, 1980), whilst McKay & Jenkin (1970b) and Paterson & Stewart (1974) have shown that phagocytosis in *P. bicarinatus* and *H. americanus* is temperature dependent and therefore utilizes energy.

In vertebrates (particularly mammals), the respiratory burst is a well documented intracellular mechanism in which stimulation of the phagocyte membrane by non-self molecules (such as LPS, ß-1,3 glucans and surface proteins of parasites) results in the production of reactive oxidizing agents that have a powerful antimicrobial activity. The mammalian respiratory burst involves a membrane-associated enzyme (NADPH oxidase) which catalyzes the conversion of molecular oxgen to superoxide ions (Babior *et al.*, 1973). Superoxide ions either spontaneously dismutate or are catalyzed by superoxide dismutase (SOD) to form hydrogen peroxide (Fridovich, 1978), which is itself microbicidal (Babior *et al.*, 1973; Nathan *et al.*, 1979). More recently the

biochemistry of the respiratory burst has also been investigated in fish (for example, Chung & Secombes, 1988; Secombes *et al.*, 1988).

Invertebrate studies of respiratory burst have produced conflicting results. Anderson *et al.* (1973) working on insects, and Cheng (1976) working with molluscs have reported that haemocytes from these animals are incapable of producing superoxide ions or other reactive oxygen moieties. On the other hand, evidence is accumulating that haemocytes from a variety of molluscan species do indeed produce both hydrogen peroxide and superoxide ions (Dikkeboom *et al.*, 1988; also Adema *et al.*, 1991 for review). Respiratory burst has also been found in an echinoderm (*Strongylocentrotus nudus*) by Ito *et al.*, (1992) and the first report of respiratory burst in crustacean hyaline cells has been made by Bell & Smith (1993a, in press) working with separated cells from the haemolymph of the shore crab *C. maenas*. Bell & Smith have also shown respiratory burst in two other crustaceans, *Galathea strigosa* and *Nephrops norvegicus* (1993b, submitted).

Although information relating to intracellular events of crustacean phagocytes is limited, it is possible that they share many features in common with the process in vertebrates since phagocytosis is likely to have been been highly conserved across the whole spectrum of animal phyla. A comparison of invertebrate and vertebrate phagocytosis is given in Anderson (1975) and it seems probable, from the evidence available, that for vertebrates and invertebrates alike, phagocytosed material is degraded by various hydrolytic enzymes that are released into the phagosome after fusion of the phagosome with lysosomal vacuoles.

1.4.4. Encapsulation and nodule formation

Encapsulation and nodule formation are host responses which have been recorded in a large number of invertebrates, including annelids, molluscs, insects, crustaceans and echinoderms (see Ratcliffe *et al.*, 1985 for review.). Both arise in response to the

presence of biotic or abiotic non-self material in the haemocoel, and generally occur when particles are too large or too numerous to be phagocytosed (Ratcliffe *et al.*, 1985). The two events are not dissimilar and involve the sequestering of non-self material in accumulations of haemocytes which eventually show evidence of melanization.

Nodule formation is also termed haemocyte clumping, especially in crustaceans, and detailed study of this has been carried out in *C. maenas* by Smith & Ratcliffe (1980a,b) and White & Ratcliffe (1982). In *C. maenas,* haemocyte clumping occurs in response to the presence of large numbers of bacteria in the haemocoel. The bacteria are rapidly removed from the circulation and localized in haemocyte clumps in the gills and hepatopancreatic sinuses (Smith & Ratcliffe, 1980a,b; White & Ratcliffe, 1982) where they adhere to the surface of granular haemocytes which then aggregate and form clumps. Granular cell exocytosis occurs and the cell clumps enlarge by the peripheral accumulation of haemocytes which become organized in concentric layers. The central core of the haemocyte clump is often necrotic and sometimes heavily melanized (Smith & Ratcliffe, 1980b). White & Ratcliffe (1982), using fluorescence-labelled bacteria, have shown significant reduction of intact bacteria within haemocyte clumps, which implies that antibacterial activity is occurring. It has already been noted in section 1.4.3 that levels of phagocytosis in crustaceans are generally low, so haemocyte clumping is an important adjunct to phagocytosis for efficient clearance of large numbers of bacteria.

Encapsulation, on the other hand, appears to be a response designed to immobilize parasites such as nematodes and large protozoans which are too large to be phagocytosed (Ratcliffe *et al.,* 1985). Mature capsules are very similar to nodules and Ratcliffe *et al.* (1985) suggest that the sequence of events leading to the development of these structures is identical.

1.4.5. Cytotoxicity

Cytotoxic reactions have been reported for a wide range of marine invertebrates, including sponges, coelenterates, sipunculids, annelids, molluscs, crustacea, echinoderms and tunicates (see review by Ratcliffe *et al.*, 1985). The necrosis of incompatible tissue types from adjacent colonies of sponges, coelenterates and tunicates is an example of cytotoxicity arising from the recognition of non-self, and has already been described in section 1.4.1.

However, invertebrate cytotoxic responses are not unique to colonial animals and have been observed in relation to the freely circulating blood cells of animals such as annelids and solitary tunicates (Ratcliffe *et al.*, 1985). As with vertebrates, the effect is dependent on cell-cell contact in the majority of cases, although an exception can be found in *M. edulis* in which cytotoxicity appears to be mediated by factors released into the surrounding medium (Wittke & Renwrantz, 1984). In some cases cytotoxicity is inhibited by a variety of sugar moieties (Decker *et al.*, 1981) which gives further support to the notion that lectins may function in invertebrate recognition (see section 1.4.1).

Reports of cytotoxicity in crustaceans are limited, although early work by Tyson & Jenkin (1974b) demonstrated that haemocytes from the crayfish *P. bicarinatus* are cytotoxic for various tumour cell lines. Tyson & Jenkin (1974b) also showed that the cytotoxic response is mediated by trypsin-labile factors associated with the membrane of the haemocyte. More recently, Söderhäll *et al.* (1985) have shown that *Astacus astacus* haemocytes are strongly cytotoxic towards mammalian tumour and non-tumour cells *in vitro* and propose a connection between this and the activation of the proPO cascade. Their proposal is based on the fact that although *Astacus* haemocyte lysate supernatant (HLS), containing the proPO system, is not cytotoxic to mammalian cells, nor are granular and semi-granular cells which have been depleted

of proPO-containing granules by calcium ionophore treatment (Söderhäll *et al.*, 1985). Thus it would seem that the cytotoxicity directed at mammalian cells not only requires the proPO system but also requires the presence of intact haemocytes. Activation of proPO in HLS, however, does cause lysis of *Astacus* semi-granular cells although mammalian target cells remain unaffected.

The precise role of the proPO system in relation to cytotoxicity remains uncertain, and whether cytotoxicity and microbial killing (see section 1.4.11) are executed through the same mechanism is unknown (Söderhäll & Smith, 1986a). Certainly for vertebrates it would seem that the mechanisms for lysis of tumour cells and microbicidal destruction are similar but not identical (Adams, 1982).

1.4.6. Agglutinins

Factors that bind to and cause the aggregation or agglutination of foreign particles have been reported for many invertebrate species, from sponges to urochordates (see reviews by Schapiro, 1975; Cooper & Lemmi, 1981; Cooper, 1985; Ratcliffe *et al.*, 1985; Amirante, 1986). They have been most extensively investigated in molluscs for which a wide range have been described (Renwrantz, 1986 - review).

In crustaceans, agglutinins have been found against vertebrate erythrocytes (see for example Cushing, 1967; Cohen, 1968; McKay *et al.*, 1969; Faglioni *et al.*, 1971; Miller *et al.*, 1972; Pauley, 1973; Hall & Rowlands, 1974a,b; Paterson *et al.*, 1976; Cornick & Stewart, 1973; Smith & Ratcliffe, 1978; Huang *et al.*, 1981; Kamiya *et al.*, 1987; Ravindranath & Paulson, 1987; Ratanapo & Chulavatnatol, 1990), bacteria (Cornick & Stewart, 1968a,b, 1975; Miller *et al.*, 1972; Huang *et al.*, 1981), invertebrate sperm (Tyler & Scheer, 1945; Smith & Goldstein, 1971), protozoans (Bang, 1962, 1967) and other cells (Tyler & Metz, 1945) (Table1.2). These agglutinins do not appear to be ubiquitous among crustaceans and when they do occur the titres are frequently low when compared with other invertebrates (Table 1.2; also

Table 1.2

Table1.2. Characteristics of crustacean agglutinins

Species	Test Particle	Highest Titre	Requirement for Divalent Cations	Heat Stability	Mol Wt (kDa)	Source	References
Homarus americanus	rbc[1] Bacteria	512 512	Ca^{++} for heat stability at pH 6-9	Stable below 56°C Freeze stable	55 (subunits)	Serum	Cornick & Stewart, 1968a Cornick & Stewart, 1973 Hall & Rowlands, 1974a Paterson *et al.*, 1976
Homarus vulgaris	rbc[1]				200	Haemocyte lysate & whole haemolymph	Durliat & Vranckx, 1989
Panulirus interruptus	rbc[1] Sperm[2]	256 (serum) 256 (serum) 512 (plasma)		Stable below 56°C	Large	Serum Plasma	Tyler & Metz, 1945 Tyler & Scheer, 1945 Cushing *et al.*, 1963
Cardiostoma guanhumi	Sperm[2]					Whole haemolymph	Smith & Goldstein, 1971
Carcinus maenas	rbc[1]	64				Plasma	Smith & Ratcliffe, 1978
Callinectes sapidus	rbc[1]	64	Yes	Stable below 50°C	150	Serum Microsomal membrane	Pauley, 1973, 1974 Vasta & Cassels, 1983 Cassels *et al.*, 1986
Maia squinado	Protozoa[3]					Serum	Bang, 1962
Geryon quinquedens	Bacteria	2				Serum	Cornick & Stewart, 1975
Chionoectes opilio	Bacteria	64				Serum	Cornick & Stewart, 1975
Birgus latro	rbc[1]	512				Serum	Cohen, 1968 Vasta & Cohen, 1984

Cancer irroratus	Bacteria [4]					Serum	Cornick & Stewart, 1968b
Graspus strigatus	rbc[1]	8				Serum	McKay *et al.*, 1969
Cancer antennarius	rbc[1]		Ca^{++} & Mg^{++}	Stable room temp for 5h. Freeze stable	70 (subunit 37)	Serum	Ravindranath *et al.*, 1985 Ravindranath & Paulson, 1987
Astacus leptodactylus	rbc[1]					Haemocyte lysate & whole haemolymph	Durliat & Vranckx, 1989
Parachearaps bicarinatus (= *Cherax destructor*)	rbc[1]	128		Inactivated at 57°C		Serum	McKay *et al.*, 1969 McKay & Jenkin, 1970a
Procambarus clarkii	rbc[1] Bacteria	256 16		Stable below 60°C Inactive at 70°C	>150	Serum	Miller *et al.*, 1972
Squilla mantis	rbc[1]	128	Ca^{++} & Mg^{++}	Inactivated at 65°C	193	Plasma	Amirante & Basso, 1984
Macrobrachium rosenbergii	rbc[1]	256				Serum	Vasta *et al.*, 1983
Penaeus stylirostris	rbc[1]	4				Serum	Vargas-Albores *et al.*, 1992
Penaeus monodon	rbc[1]		Ca^{++} & Sr^{++}	Stable below 55°C Inactive at 65°C	420 (subunit 27)	Whole haemolymph Ovary, testis, muscle & hepatopancreas	Adams, 1991 Ratanapo & Chulavatnatol, 1990
Triops cancriformis	rbc[1]			Stable below 56°C		Whole haemolymph	Cenini, 1983
Megabalanus volcano	rbc[1]	1024	Ca^{++}	Stable below 60°C inactivated at 80°C	116 (subunit 23)	Plasma	Kamiya & Shimizu, 1981 Kamiya & Ogata, 1982 Kamiya *et al.*, 1987

[1]rbc: vertebrate erythrocytes. [2]sperm: invertebrate sperm. [3]protozoa: *Anophrys sarcophaga*. [4]bacteria: *Gaffkya homari* (= *Aerococcus viridans* (var.) *homari*.

the surveys by Brown *et al.*, 1968 and Faglioni *et al.*, 1971 and review by Smith & Chisholm, 1992). Several agglutinins may be present in any one species but levels of activity may differ considerably between individual animals (Adams, 1991). So far, approximately forty crustacean species, chiefly decapods, have been shown to possess haemolymph agglutinins (Table 1.2 and also surveys by Brown *et al.*, 1968 and Faglioni *et al.*, 1971), but there seems to be no clear trend in their occurrence and distribution within the group.

Apart from the work of Cohen (1968), Vasta & Cohen (1984) and more recently, Vargas-Albores *et al.* (1992), there has been no proper evaluation of age-related changes in levels of agglutinin activity in crustaceans. Cohen (1968) and Vasta & Cohen (1984) have reported higher titres of agglutination in larger (older) specimens of the coconut crab (*Birgus latro*) compared to smaller (younger) ones, and Vargas Albores *et al.* (1992) have made similar findings in *Penaeus stylirostris*. Likewise, variations in such activity with respect to the moult cycle are poorly reported although Ghidalia *et al.* (1975) noted no differences in haemagglutinin levels in *Macropipus puber* from moult stage C2 to D2. Muramoto *et al.* (1991), have found that the agglutinins in the barnacle *Megabalanus rosa* are maximally active in early summer, but in general, seasonal changes in agglutinin levels have been investigated rarely. In addition, there is a conspicuous lack of information with respect to agglutination of bacteria, protozoans and other cells or microorganisms, whereas haemagglutination has been extensively studied.

Where found, crustacean agglutinins appear to occur naturally in the plasma or serum although enhanced titres following prior exposure of the host to the test materials have been reported for *Callinectes sapidus* by Pauley (1973) and *Penaeus monodon* by Adams (1991). In both instances the effect of immunization is small, short-lived and non-specific. There is little evidence to suggest that crustacean agglutinins are predominately inducible and none to suggest that they represent an

adaptive element of the host response or could be exploited in the development of vaccines.

As far as agglutination of micro-organisms is concerned, there can be no doubt that these factors must assist in the sequestration of invasive organisms from the haemolymph, thereby facilitating their interaction with the circulating blood cells and preventing the spread of potentially infective agents around the body. It is therefore surprising to find little evidence correlating the occurrence of agglutinins with disease resistance in crustaceans. In an early study, Bang (1962) noted that the spider crab, *Maia squinado* has potent agglutinating activity against the ciliate parasite, *A. sarcophaga.* Unlike the shore crab *C. maenas* (the normal host of *A. sarcophaga), M. squinado* is usually resistant to the protozoan and resistant spider crabs always agglutinate the parasite (Bang, 1962). Interestingly, however, a few individuals of *M. squinado* show susceptibility to *A. sarcophaga,* and in these animals agglutinating activity is absent (Bang, 1962). Likewise, with the lobster, *H. americanus,* Cornick & Stewart (1968a) failed to detect serum agglutinins in the serum against the bacterial pathogen *G. homari* (= *Aerococcus viridans* (var.) *homari*) but recorded activity against several other non-pathogenic bacteria. On the other hand, serum from the Atlantic crab, *Cancer irroratus,* for which *G. homari* is mildly pathogenic (Cornick & Stewart, 1975; Newman & Feng, 1982), was found to display weak agglutinating activity against *G. homari* (Cornick & Stewart, 1968b) while strong activity was demonstrated in the snow crab, *Chionoecetes opilio,* which is highly resistant to the bacterium (Cornick & Stewart, 1975). Paradoxically, serum from the red crab, *Geryon quinquedens,* fails to agglutinate *G. homari* despite its resistance to infection by this micro-organism (Cornick & Stewart, 1975). Clearly, while antimicrobial agglutinins contribute to protection of the host against disease, resistance does not necessarily reside with any single aspect of the defence networks.

The function of the haemagglutinins is less obvious. Typically, they are inhibited by particular sugars and this affinity for polysaccharides has encouraged several workers to consider them as lectins (Table 1.3). Lectins are defined by the Nomenclature Committee of the International Union of Biochemists as carbohydrate binding proteins of non-immune origin that agglutinate cells or precipitate polysaccharides or glycoconjugates (Liener *et al.*, 1986). They are usually heat-sensitive proteins or glycoproteins with a polyvalent configuration and binding affinity for specific cell surface polysaccharides (Yeaton, 1981a). They encompass a huge class of molecules and have been found in plants, micro-organisms and vertebrates as well as invertebrates (Yeaton, 1981a,b; Liener *et al.*, 1986). Specifically, they do not include carbohydrate binding proteins of known enzymic function, carbohydrate transport proteins or carbohydrate specific antibodies (Goldstein & Hayes, 1978). Partly because of their value as biochemical tools, interest in lectins has increased enormously in the past decade and a wide variety of molecules from a range of sources have been ascribed the status of lectins, albeit inappropriately on occasions. As pointed out by Goldstein *et al.* (1980), an important diagnostic feature of true lectins, which distinguishes them from glycosidases and glycotransferases, is their possession of two or more sugar specific binding sites which enables them to agglutinate foreign cells. Thus, in accordance with the definition cited above, some crustacean haemagglutinins may be reasonably considered as lectins (Table 1.3). Unfortunately, in other cases, the biochemical character of the agglutinin has not been properly defined so there may be insufficient grounds for adopting the terminology. This confusion in nomenclature complicates our understanding of haemagglutinin biochemistry and the indiscriminate use of the term lectin may carry unsubstantiated functional implications.

Lectins appear to serve a variety of biological functions (Lis & Sharon, 1986; Liener *et al.*, 1986) and in molluscs, strong evidence has been presented in favour of

Table 1.3

Table 1.3. Characteristics of crustacean lectins

Species	Principle Erythrocyte Binding Reactions	Sugar Specificity	Mol Wt. (kD)	Divalent Cation Requirement	References
Crabs					
Cancer antennarius	Horse, rabbit, rat, mouse	9-*O* - NANA 4-*O* - NANA	70 (dimer)	Ca^{++} & Mg^{++}	Ravindranath *et al.*, 1985 Ravindranath & Paulson, 1987
Callinectes sapidus	Human A, horse, rabbit, rat	NANA GalNAc GlcNAc ManNAc *N*-acetylmuramic acid *N*-acetyl glutamic acid			Cassels *et al.*, 1986
Birgus latro	Human ABO, monkey, birds, rodents	NANA			Cohen, 1968 Vasta & Cohen, 1984
Lobster					
Homarus americanus	Human (for LAg 1) Mouse (for LAg1 & LAg2)	NANA (LAg 1) GalNAc (LAg2)	55 (subunits)	Ca^{++}	Cornick & Stewart, 1973 Hall & Rowlands, 1974 a,b Hartman *et al.*, 1978 Goldenberg & Greenberg, 1983

Shrimps & Prawns					
Squilla mantis	Human AO	GalNAc (anti-A) Fucose (anti-H)	193	Ca^{++} & Mg^{++}	Amirante & Basso, 1984
Penaeus monodon	Human O	NANA	420	Ca^{++}	Ratanapo & Chulavatnatol, 1990
Macrobrachium rosenbergii	Human ABO, horse, rat, duck, goose	NANA			Vasta *et al.*, 1983
Barnacles					
Megabalanus volcano	Human ABO, rabbit	Lactose Fetuin	116	Ca^{++}	Kamiya *et al.*, 1987
Megabalanus rosa	Human , rabbit (murine tumour cells)	Galactose	330 (BRA1) 140 (BRA2) 64 (BRA3)	Ca^{++}	Muramoto *et al.*, 1985

Abbreviations used are: NANA, *N*- neuraminic acid; GalNAc, *N*- acetylgalactosamine; GlcNAc, *N*- acetylglucosamine; ManNAc, *N*- acetylmannosamine

LAg, lobster agglutinin; BRA, *Megabalanus rosa* agglutinin.

their role as recognition molecules (see reviews by Ratcliffe *et al.*, 1985 and Renwrantz, 1983, 1986). However, information that they serve an equivalent function in crustaceans is either lacking or largely equivocal. With the freshwater crayfish, *Cherax destructor* (formerly *P. bicarinatus*), Tyson & Jenkin (1973, 1974a) reported significantly enhanced rates of clearance of injected bacteria and *in vitro* phagocytosis following pre-incubation of the foreign particles in crayfish serum. The serum is known to contain agglutinins against vertebrate erythrocytes and their adsorbtion from the serum was found to abolish its opsonic properties (McKay & Jenkin, 1970a). These findings were originally interpreted as evidence for the presence of functional analogues of vertebrate immunoglobulins in invertebrate body fluids (McKay & Jenkin, 1970a; Tyson & Jenkin, 1973, 1974a). However, when Hall & Rowlands (1974b) attempted to show that the haemagglutinins purified from *H. americanus* behave as an opsonin, they obtained only low rates of phagocytosis in their *in vitro* assays despite the presence of the agglutinins. More recently, by fractionization of lobster haemolymph on Sephadex G-200, Goldenberg & Greenberg (1983) have found the opsonizing and agglutinating activities of *H. americanus* haemolymph to be separate and thus it is unlikely that this particular agglutinin acts opsonically.

Biochemically, only a few crustacean agglutinins have been characterized. In general, these studies have revealed that most are proteinaceous, heat labile and dependent upon divalent cations, usually calcium ions (Table 1.2). N-acetylneuraminic acid (sialic acid) specific agglutinins have been reported to occur in the haemolymph of several taxonomically separate crustaceans, including prawns, barnacles and crabs (Table 1.3). A small number of these have been purified, revealing the presence of multiple agglutinins in several species (Hall & Rowlands 1974a,b; Hartman *et al.*, 1978; Amirante & Basso, 1984; Cassels *et al.*, 1986), but data on molecular weights, sedimentation coefficients, amino acid and carbohydrate

composition are still scant and sequencing has been carried out only occasionally (Table 1.3).

The presence of multiple agglutinins in invertebrates has sometimes been taken to support the view that these molecules function in recognition and/or are immunoglobulin precursors. However, the paucity of information about their biochemical structure makes it difficult to assess their biological significance and to evaluate the phylogeny of these molecules across the invertebrates as a whole. A particularly detailed study has recently been made of the structure and amino acid sequence of the agglutinins in the acorn barnacle, *M. rosa,* by Muramoto and his co-workers (see Kamiya & Ogata, 1982; Muramoto *et al.*, 1985; Muramoto & Kamiya, 1986, 1990a,b). Up to three galactose-binding agglutinins have been identified for *M. rosa*, each with a different molecular weight, designated BRA-1 (M_r 330,000), BRA-2 (M_r 140,000) and BRA-3 (M_r 64,000). Both BRA-1 and BRA-2 are composed of identical subunits (M_r 22,000) which are cross linked by interchain disulphide bonds, while BRA-3 is composed of four identical subunits (M_r 16,000) of 138 amino acids. Analysis of their amino acid sequences has revealed that they show marked homology to other invertebrate agglutinins as well as to C-type (calcium dependant) lectins of vertebrates, but not to plant lectins or vertebrate S-type agglutinins. This suggests that vertebrate and invertebrate agglutinins might have a common ancestral gene but until more invertebrate agglutinins are examined, the phylogeny of these molecules remains speculative (Muramoto & Kamiya, 1990a). It is interesting to note that although the presence of highly conserved cysteine residues (involved in the formation of disulphide bridges) in the barnacle agglutinin is reminiscent of the heavy chain segments of human immunoglobulins, no similarity exists with immunoglobulin variable regions (Muramoto & Kamiya, 1986, 1990b).

Perhaps because of the desire to find a link between the recognition molecules of vertebrates and invertebrates, there has been an assumption by nearly all investigators

that agglutinins form a major part of the humoral, as opposed to the cellular, defences in invertebrates. However, the origin of these factors in most crustacean species has not been established and in a few instances they appear to be associated with the cell membranes (Vasta & Cassels, 1983; Cassels *et al.*, 1986). Moreover, in many studies it is serum (i.e. the fluid remaining after clotting) rather than plasma, which has been tested for agglutinating activity. As already observed, crustacean haemocytes are remarkable for their extreme sensitivity to endotoxin and other non-self materials and rapidly undergo exocytosis or lysis *in vitro* (Smith & Söderhäll, 1983a; Söderhäll *et al.*, 1986) (see section 1.4.2). The accompanying activation of previously cell bound enzymes by the foreign molecules releases a number of 'sticky' proteins (Söderhäll *et al.*, 1983) which could bring about the extracellular aggregation of the non-self particles *in vitro*. Even where plasma fractions have been examined, adequate precautions against haemocyte lysis during sample preparation have not always been taken, making it difficult to determine the extent to which any agglutinating effect could be cell-derived.

Overall, the site(s) of agglutinin synthesis in crustaceans remains obscure. In some cases they have been found in association with the cells but in other instances they have not. For example, Cornick & Stewart (1973) have noted that agglutinin activity is associated with the haemocytes in *H. americanus*, and Amirante & Basso (1984) have demonstrated, with monoclonal antibodies, that the haemagglutinin in *S. mantis* is present on the membranes of the granular haemocytes as well as free in the plasma. More recently, Ratanapo & Chulavatnatol (1990) have reported that the agglutinin in *P. monodon* occurs in the ovary, testis, hepatopancreas and muscle as well as the haemolymph. By contrast, Smith & Söderhäll (1983a) have found that in *C. maenas* weak haemagglutinating activity toward sheep, horse and human erythrocytes resides in the serum, whilst none is present in lysate supernatants of the haemocytes. The absence of haemagglutinating activity in these extracts does not

necessarily preclude the location of agglutinins on the haemocyte surface but shows that in this species they are not present in a soluble form within the cells.

1.4.7. Clotting

Clotting is an essential process in all coelomate animals and serves to seal wounds, stem blood loss and provide some protection from potentially harmful foreign agents. In most invertebrates this involves aggregation of circulating cells but in the arthropods there is additional plasma gelation which is mediated by factors released from the haemocytes (Grégoire 1971). The phenomenon has been most extensively studied in the chelicerates, mainly *L. polyphemus* and its close relatives (see for example Iwanaga, 1993), and several excellent accounts of clotting in arthropods have been published (Durliat, 1985; Levin, 1985; Bohn, 1986; also review by Ratcliffe *et al.*, 1985).

In most crustaceans clotting is mediated by coagulogens which are present in the plasma and also compartmentalized in the circulating cells. The plasma factor bears a similarity to vertebrate fibrinogen and is converted to covalently linked polymers of coagulin by Ca^{++} dependent transglutaminase (Durliat, 1985). A clotting protein from crayfish plasma has recently been isolated and characterized by Kopacek *et al.* (1993, in press) and has a molecular mass close to that reported for lobster fibrinogen (Doolittle & Fuller, 1972). The cell factor operates in a different way and is converted to a gel by a serine protease pro-clotting enzyme which may be triggered by lipopolysaccharide (LPS) or ß 1, 3-glucans (Durliat, 1985; Durliat & Vranckx, 1989). In this way it has features in common with the proPO system (Söderhäll, 1981, 1982; Söderhäll & Smith, 1986b) and is probably discharged from the cells as a consequence of non-self stimuli (Smith & Söderhäll, 1983b; Johansson & Söderhäll, 1985). Reaction of the cell factor with the plasma factor results in clot formation.

Similar events occur in insects where the plasma and cell coagulogens are highly partitioned with no evidence of plasma factor in the haemocytes (Barwig & Bohn, 1980; Bohn *et al.*, 1981; Bohn, 1986). In macruran crustaceans, the plasma factor cannot gel without haemocyte clumping (Durliat, 1985). However, haemocyte lysate supernatants containing only cellular coagulogen are able to undergo a clotting reaction quite independently of the plasma, providing that protease activity has been induced by the appropriate non-self molecules (Söderhäll, 1981; Durliat, 1985; Durliat & Vranckx, 1989). Thus clotting furnishes a good example of the way in which haemocytes and plasma factors interact to bring about an effective host response.

One exception to this pattern in the crustaceans is found in the parasite, *Sacculina carcini*, which has a clear, acellular, non-circulating coagulable liquid (presumably plasma) in the space between the basilar membrane and mantle (Levin, 1967). This fluid forms a gel and is capable of immobilising planktonic ciliates without cellular intervention (Levin, 1967). Bacteria or bacterially-derived endotoxins also stimulate gelation of the plasma in this animal, although exhaustion by these agents usually results in bacteraemia and death of the sacculine, often with the subsequent demise of its decapod host (Levin, 1967).

The crustacean system is in contrast to that of the Limulidae, in which all the factors required for coagulation of the blood seem to be located in the circulating amoebocytes (Levin, 1985). The main clotting protein comprises a single polypeptide chain which is converted to a non-covalently cross-linked gel through a serine protease (Durliat, 1985; Levin, 1985). This protease is similar to the one present in the prophenoloxidase cascade of other arthropods and may be activated by one or other of two pathways: one stimulated by LPS and the other by ß 1,3-glucans (Kakinuma *et al.*, 1981; Morita *et al.*, 1981). Other similarities exist between the clotting pathways of chelicerates and the prophenoloxidase activating system of

crustaceans but there is a striking difference in the absence of the terminal component, phenoloxidase, from the *Limulus* system.

1.4.8. Precipitins

Precipitins have been reported only occasionally for invertebrates, and in the Crustacea their presence is noted only in the older literature. Interest in these factors was probably connected with early ambitions to discover antibody-like substances in lower animals but, in parallel with the changing view of non-self recognition processes in invertebrates, enquiry into precipitins has declined. One of the first descriptions for crustaceans was by Osawa & Yambuui (1963) who found weak precipitin activity in *Cambarus clarkii,* following inoculation with sheep erythrocytes. Later, Stewart & Foley (1969) recorded a naturally occurring precipitin to fluorescein-labelled bovine serum albumin in *H. americanus.* In this latter case, the factor responsible was found to be dialysable and stable to 50°C, but to be present at low titres (Stewart & Foley, 1969). Furthermore, as there was no accumulation of label within the circulating cells, there did not appear to be any direct cellular intervention in the removal of this protein from the blood (Stewart & Foley, 1969).

More recently, in an elegant series of investigations, Clem and his co-workers have shown that in *C. sapidus,* radioactively labelled proteins injected into the haemolymph are very rapidly cleared from the circulation in a dose-independent manner which does not rely on factors present in the plasma (McCumber & Clem, 1977; Clem *et al.*, 1984). However, the response does not seem to involve intervention of the circulating cells as there is no binding of the proteins to the haemocytes, and extirpation of 99 % of the haemocytes fails to impair protein clearance (Clem *et al.*, 1984). Furthermore, immunization has no stimulatory effect on the rate of clearance of secondary challenge; instead, exposure of the experimental animals to relatively large doses of the protein immediately prior to injection of the

labelled proteins tends to slow down their removal from the blood (Clem *et al.*, 1984). Because of the absence of plasma factors and lack of haemocytic involvement, the mechanism for the elimination of non-self proteins in this animal is unclear. McCumber & Clem (1977) also found that the proteins become localized in the gills and it is possible that fixed cells (such as the nephrocytes) in these structures could play some role in the recorded protein removal (Clem *et al.*, 1984). Evidence that a fixed reticuloendothelial-type network exists in crustaceans has been presented by Smith & Ratcliffe (1981) who demonstrated that whilst the nephrocytes in *C. maenas* are not actively involved in the sequestration of injected bacteria from the blood, they accumulate debris generated during the formation of haemocyte clumps (nodules) *in vivo*. Confirmation of the role of the nephrocytes in protein elimination awaits identification of the appropriate receptors on their cell surface, but, once again, it seems that, in crustaceans, there might be a cellular aspect to an erstwhile humoral immune response.

1.4.9. Cytokine-like factors

Cytokines (or, as they are more usually known in connection with vertebrate immune systems, lymphokines) are non-antibody proteins, produced, in mammals, by activated lymphocytes. They assist in homeostatic regulation of the immune system and facilitate integration of the immune response with other body processes, by, for instance, controlling the production and maturation of the different cell types, influencing neuro-endocrine function, mediating acute phase responsiveness, affecting cell movement or displaying cytotoxic properties of their own (Adams, 1982).

It is debatable whether equivalent factors can be said to exist in invertebrates, mainly because of the constraint their definition imposes, being strongly biased towards vertebrates. In terms of blood cell-derived factors that mediate, promote or

modify immune reactivity, there are a few cases where cytokine-like activity can be reasonably claimed for lower animals, principally in starfish (Leclerc *et al.*, 1981; Prendergast *et al.*, 1983; see also review by Ratcliffe *et al.*, 1985).

As regards crustaceans, there is, unfortunately, very little information available. There are a number of factors produced by activated haemocytes (see section 1.4.2 for details) that have a controlling influence on the host defences, usually in connection with the induction of the prophenoloxidase system - for example, the glucan-binding molecule of crayfish plasma (Duvic & Söderhäll, 1990) enhances the activation of haemocyte-derived protease and prophenoloxidase by laminarin, but has no protease or phenoloxidase properties of its own and is not an agglutinin or lectin (Duvic & Söderhäll, 1990). In amino acid composition and biological activity, this glucan binding factor resembles an equivalent protein in insect plasma (Ochiai & Ashida, 1988; Söderhäll *et al.*, 1988) and is thought to assist in recognition by triggering the phenoloxidase cascade, not through phenoloxidase directly, but through the activating serine protease (Duvic & Söderhäll, 1990). However, it remains largely a matter of opinion as to whether this, or other proPO cascade-related proteins, may be designated cytokines.

Although chemotactic agents or substances that stimulate haemopoiesis or direct neuro-endocrine function have not been found in crustaceans, it is not implausible that such molecules might occur, especially as activation of the prophenoloxidase system is known to involve proteolytic cleavages which generate small peptides (see review by Söderhäll & Smith, 1986b).

1.4.10. Modulators

There has been little attempt to determine how the agglutinating, lytic or biocidal agents in crustacean blood are regulated, despite the obvious need for modulating molecules to prevent inappropriate or massive intravascular triggering of the defence

system. One factor that has been found in crayfish plasma is the high molecular weight proteinase inhibitor already mentioned in section 1.4.2 (see also Hergenhahn & Söderhäll, 1985) and which resembles α_2-macroglobulin (α_2-M) of vertebrates (Hergenhahn *et al.*, 1988; Hall *et al.*, 1989). It is known that in vertebrates, α_2-M has the effect of 'caging' proteinases, thereby reducing their activities towards large substrates but leaving their proteolytic effect on small substrates intact (Sottrup-Jensen *et al.*, 1989). The crayfish factor, like that of mammalian α_2-M, inhibits the activity of trypsin and chymotrypsin and contains a basic monomeric M_r polypeptide subunit of high molecular weight (about 155 kDa in *Pacifastacus leniusculus*) (Hergenhahn *et al.*, 1987). This high M_r inhibitor also blocks the serine protease associated with prophenoloxidase activation and is believed to serve in a regulatory capacity for the enzyme cascade (Hergenhahn *et al.*, 1987). In crustaceans, α_2-M-like activity has been described mainly for the plasma, but in the horseshoe crabs (Limulidae) it has also been found in association with the circulating amoebocytes (Armstrong *et al.*, 1985, 1990). At the moment, it is not known whether this also obtains for decapods or is a situation unique to the chelicerates because of their particular form of clotting.

1.4.11. Killing factors

A wide variety of substances that kill foreign cells or micro-organisms have been described for invertebrates (see reviews by Cooper & Lemmi, 1981; Cooper, 1985; Ratcliffe *et al.*, 1985). They are ubiquitous throughout the Invertebrata and may act against bacteria, fungi, viruses, protozoans or other foreign cells. In general, these factors are non-specific and comprise a heterogeneous collection of molecules. Their killing effects range from general disinfection, as with the pigment, echinochrome A from echinoderms (Service & Wardlaw, 1985), to lysis of defined peptidoglycan bonds, as is the case for lysozyme. Some, such as the didemnins from tropical sea squirts (Rinehart *et al.*, 1981,1983) and the battery of potent bactericidins (cecropins,

defensins, diptericins and apidaecins) from insects (see reviews by Boman, 1986; 1991), represent unique families of antimicrobial molecules which may have potential therapeutic value as antibacterial, antiviral or antineoplastic agents.

In crustaceans, killing factors have been reported relatively infrequently and they have tended to receive rather less scrutiny than the agglutinins. They encompass factors that are effective against viruses (McCumber *et al.*, 1979) bacteria (Evans *et al.*, 1968, 1969a,b; Acton *et al.*, 1969: Weinheimer *et al.*, 1969a; Stewart & Zwicker, 1972; Mori & Stewart, 1978a; Söderhäll & Smith, 1986b; Adams, 1991) or fungi (Nyhlén & Unestam, 1980) (Table 1.4) but ones against protozoans or other parasitic animals have seldom been described. Haemolysins have also been reported in a few species, for example, in *M. squinado* (Cantacuzène, 1920), *Panulirus argus* (Weinheimer *et al.*, 1969b) and *Triops cancriformis* (Cenini, 1983). However, little is known about these factors or the extent to which they contribute to antimicrobial defence.

With respect to antiviral activity, Taylor *et al.* (1964) were the first to demonstrate that crustaceans are able to clear viruses from the haemolymph. Later, McCumber & Clem (1977) showed that in *C. sapidus* clearance is accompanied by the deposition of these agents in the tissues, although the final destination of the sequestered virus is variable. T_2 and T_4 bacteriophage, for example, are cleared to the hepatopancreas whereas poliovirus is deposited in the gills (McCumber & Clem, 1983). The response seems to be augmented by a neutralizing factor in the haemolymph, which is unrelated to either haemocyanin or the haemagglutinin for mouse erythrocytes (McCumber *et al.*, 1979). Significantly, plasma is more effective than the serum in neutralizing the bacteriophages (McCumber *et al.*, 1979) and preliminary characterization has established that in *C. sapidus*, the factor responsible is a polymer of non-covalently linked subunits, each with a molecular weight of *circa* 80 kDa (McCumber *et al.*, 1979). However, understanding of anti-viral defence in

Table1.4. Antibacterial, antiviral and fungitoxic factors in crustacean haemolymph

Species	Test Organisms	Inducibility	Requirement for Divalent Cations	Heat Stability	Comments	References
Panulirus argus	Gram-negative bacteria	yes	no	Stable below 60°C Inactive at 65°C Freeze stable -25°C	Not agglutination Enhanced 2° & 3° responses	Evans *et al.*, 1968 Evans *et al.*, 1969a Weinheimer *et al.*, 1969a
Panulirus interruptus	Gram-negative bacteria	yes		Stable at 60°C Inactive at 65°C	Not agglutination 2° responses noted	Evans *et al.*, 1969b
Homarus americanus	*Pseudomonas perolens* & *Aerococcus viridans* (var.) *homari*	yes			Mainly in hepatopancreas	Acton *et al.*, 1969 Stewart & Zwicker, 1972 Mori & Stewart, 1978a,b
Callinectes sapidus	bacteriophages poliovirus		no	Stable at 56°C	Plasma more effective than serum 6-13S 80 kDa polymer	McCumber *et al.*, 1979 McCumber & Clem, 1983
Carcinus maenas	marine bacteria				Present in granular cells	Söderhäll & Smith, 1986a
Penaeus monodon	Gram-negative bacteria	yes			Partial specificity to vibrios	Adams, 1991
Pacifastacus leniusculus	*Aphanomyces astaci*				May be due to melanin or melanin precursors	Nyhlén & Unestam, 1980

crustaceans is in general rather poor and work to support and extend these preliminary findings is currently under way in this laboratory.

Bactericidins have attracted most attention, especially in crabs and lobsters (Table 1.4), probably because of the high value these animals have for the aquaculture industry. Typically, bactericidal activity in lobsters appears to be effective against a variety of bacteria, the main exception being the pathogen, *G. homari* (Cornick & Stewart, 1968a,b, 1975; Stewart & Zwicker, 1972) (Table 1.4). The lack of activity against *G. homari* undoubtedly contributes to its pathogenicity as the haemolymph provides an excellent nutritive medium for growth (Cornick & Stewart, 1968a). Bactericidins against *G. homari* are also absent from the haemolymph of the crabs (Cornick & Stewart, 1968b, 1975) which are either resistant or only mildly susceptible to the pathogen (Cornick & Stewart, 1975; Newman & Feng, 1982). Otherwise, studies have been concerned primarily with the host response to Gram-negative bacteria, including isolates from the gut of the host animal, rather than Gram-positive forms (Evans *et al.*, 1968, 1969a; Weinheimer *et al.*, 1969a; Mori & Stewart, 1978b).

In many cases, killing activity has been induced by pre-treatment of the host to live or heat-killed bacteria (Evans *et al.*, 1968, 1969a,b; Acton *et al.*, 1969; McKay & Jenkin, 1969; Weinheimer *et al.*, 1969a, Stewart & Zwicker, 1972; Mori & Stewart, 1978b; Adams, 1991) (Table1.4). However, these responses appear to have limited specificity and the time taken to reach maximum effect is highly variable, ranging from as little as 36-48h (Evans *et al.*, 1968; 1969a; Adams, 1991) to 7 days (Evans *et al.*, 1969b). So far the phenomenon has not been properly explained. Analyses of the protein composition of the plasma after stimulation have not been carried out and no work has been done to determine whether pre-exposure of the host provokes protein synthesis by the tissues, as is the case with lepidopteran insects (see review by Boman, 1986). It is well established that injection of foreign agents into the

haemocoel of crustaceans produces a marked haemocytopaenia within a few hours, and recovery of the cell number can take at least two days (Smith & Ratcliffe, 1980a,b; Smith *et al.*, 1984). The extent to which changes in the number of circulating cells (and quantities of bioactive substances released by them) are related to the induction of bactericidins has not been assessed.

Certainly, as with the agglutinins, killing does not appear to be restricted to the serum or plasma. While microbicidins occur in the haemolymph of some species (Stewart & Zwicker, 1972; Mori & Stewart, 1978a; Adams,1991), in others, they may be associated with different tissues (Mori & Stewart, 1978b). With lobsters, bactericidal activity has been found to reside predominantly in the hepatopancreas and the small amount present in the haemolymph is not associated with the haemocytes (Mori & Stewart, 1978b). It has also been reported for *H. americanus* (Stewart & Zwicker 1972) that bactericidal activity exists *in vivo* largely in an inactive form and requires co-operation with a factor within the haemocytes for full expression. By contrast, although Smith & Ratcliffe (1978) and White *et al.* (1985) have failed to detect bactericidal or bacteriostatic factors in the plasma or serum of *C. maenas*, Söderhäll & Smith (1986b) have noted strong activity in haemocyte lysate supernatants (HLS).

Biochemically, little is known about the killing factors in crustaceans. Most have no requirement for divalent cations, which distinguishes them from the agglutinins (see section 1.4.6), and the majority are inactivated above 60 °C (Evans *et al.*, 1968, 1969b; Weinheimer *et al.*, 1969a; McCumber *et al.*, 1979) (Table 1.4). The antibacterial factor in *P. argus* is additionally reported to be stable down to -25°C (Weinheimer *et al.*, 1969a). In other respects, biochemical characterization is incomplete which makes it impossible to give a satisfactory account of the mechanisms underlying bacterial killing. Furthermore, the extent to which bactericidal

activity is discrete from bacteriostasis has not always been made clear and it is uncertain whether the foreign agents are destroyed by lysis or other means.

Lysozyme, an enzyme capable of splitting the ß-1,4 glycosidic links of bacterial cell walls, exists in many invertebrate groups, especially insects and molluscs (see review by Ratcliffe *et al.*, 1985) but had not been reported for crustaceans until very recently when Fenouil & Roch (1991) produced evidence of lysozyme in six species of freshwater crayfish. Peroxidase-mediated antimicrobial pathways play an important part in antimicrobial defence in vertebrates (Klebanoff, 1975) but there appears to be no evidence for a comparable system in crustaceans (White *et al.*, 1985; Hose *et al.*, 1987).

Recently, Boman (1991) has discussed the role of peptides in antibacterial defence and has pointed out that in terms of their broad specificity, lack of reactivity against eukaryotic cells, rapid diffusion rates and relative ease of synthesis, they have unique advantages in non-specific host defence. More importantly, from a phylogenetic standpoint, there is no need for their synthesis by specialized cells and they do not require a repertoire of recognition molecules for effect. They are thus ideal candidates for primitive defence molecules. Such bacteriolytic peptides are known to occur in Lepidoptera, in the form of the cecropins and hemolin (see reviews by Boman, 1986; Boman & Hultmark, 1987); in addition similar molecules (dipteracins and apidaecins) have been identified in flies and bees respectively (1988; Casteels *et al.*, 1989; Boman, 1991). These molecules are induced by bacterial challenge and are highly potent towards both Gram-negative and Gram-positive bacteria. All have 31-39 residues, are devoid of cysteine and have a strongly basic N-terminal region and a long hydrophobic stretch in the C-terminal half (Boman, 1991). The occurrence of these factors is not restricted to insects and has now been detected in pig intestine, showing that they have been conserved during evolution and may be widely distributed within the animal kingdom (see Boman, 1991). As yet, cecropins have not

been identified in crustaceans, but it is not unreasonable to predict that they might be found, bearing in mind the close phylogenetic relationship of these animals with the insects.

The antibacterial factor in *C. maenas* seems to reside principally in the granular cells (Söderhäll & Smith, 1986b), so it is possible that antibacterial activity may be associated with the activation of prophenoloxidase. Previously, Unestam (1975) and Nyhlén & Unestam (1980) have observed the deposition of melanin around fungal hyphae in the tissues of the crayfish. Söderhäll & Ajaxon (1982) have subsequently demonstrated that quinones and melanin (products of phenoloxidase activation) are fungitoxic, thereby giving credence to the suggestion of Nyhlén & Unestam (1980) that melanin or its precursors behave as inhibitors of fungal enzymes. As previously noted (section 1.4.4), Söderhäll *et al.* (1985) have shown that *A. astacus* haemocytes, but not *A. astacus* HLS, display potent cytotoxicity towards ^{51}Cr-labelled human or mouse tumour cells *in vitro*. It is probable that a variety of killing strategies operate within the crustacean group, but it would be pertinent to determine the exact role of the prophenoloxidase system in extracellular killing and to ascertain whether components of the system have other antimicrobial properties.

1.5. Specific Aims of Study

The preceding sections of this chapter have highlighted several areas where our understanding of the crustacean immune system is incomplete. This is particularly true with regard to recognition processes, modulation of the immune response and antimicrobial activity in the haemolymph.

To redress the balance with regard to antibacterial activity, and using *Carcinus maenas* as the test animal, this thesis will explore the hypothesis that there is a

connection between the antibacterial activity in crustacean haemocytes and the proPO activating system. In addition, *in vitro* studies will be described that characterize the antibacterial factor more fully.

Carcinus maenas is an appropriate experimental animal: it is abundant in Scottish waters, forms an important component of the food chain and last but not least, each specimen provides a generous volume of haemolymph without necessitating sacrifice of the animal. Previously, *C. maenas* has been used to investigate several physiological parameters associated with haemolymph, including circulation and respiration (Depledge, 1978 & 1984; Burnett & Johansen, 1981; Lallier *et al.*, 1987), protein composition (Uglow, 1969a,b; Horn & Kerr, 1969) carbohydrate metabolism (Johnston & Davies, 1972; Johnston *et al.*, 1973; Williams & Lutz, 1975a,b), phagocytosis and nodule formation (see for example, Smith & Ratcliffe, 1978, 1980a,b). In addition, Mantel (1983) provides a good general text in relation to a number of aspects of crustacean physiology.

As already observed in section 1.4.11, there is a paucity of information with respect to the spectrum of activity, divalent cation requirement, titre and thermal stability of crustacean antibacterial factors. Work relating to this is described in Chapters 2 and 4. Chapter 3 is devoted to a study of seasonal variation in antibacterial activity and Chapter 5 to the mode of action of the antibacterial factor(s). Isolation of the factor (or factors) involved was attempted in the later stages of the study and is reported in Chapter 6. In the final chapter relating to experimental work, Chapter 7, evidence of similar activity in the haemocytes of four other disparate crustaceans is presented. In conclusion, Chapter 8 comprises a general discussion and assessment of the overall results of the work presented herein.

Chapter 2
Spectrum of Antibacterial Activity in *Carcinus maenas* Haemocytes

2.1. Introduction

Many aspects of host defence in crustaceans have been addressed at length (see Chapter 1), but the particular phenomenon of antibacterial activity (as distinct from agglutination, phagocytosis or haemocyte clumping) has received comparatively little attention. A natural starting point for this investigation of antibacterial activity in *Carcinus maenas* haemocytes was to consider the spectrum of activity, using a number of different Gram-negative and Gram-positive organisms from geographically diverse marine waters.

Bacteria were selected to comply with a number of criteria; not only were they from a variety of sources, but, in addition, most had distinctive colony formations (which distinguished them from contaminants) and were easily cultured under laboratory conditions. Also, it was considered relevant to include known marine pathogens of fish and crustaceans in the survey. One, *Aerococcus viridans* (var.) *homari* is a lobster pathogen that causes Gaffkaemia (Stewart & Rabin, 1970; Stewart, 1975). Another, *Vibrio anguillarum* is a fish pathogen that causes 'red disease' in eels (Sindermann 1968), but has also been isolated from moribund shrimp and crabs (see Johnson, 1983 for review). *Vibrio anguillarum* has also caused death of juvenile lobsters under experimental conditions and has been implicated in the occasional high mortalities found amongst commercially held blue crabs (Johnson, 1983).

Previous studies of antibacterial activity in crustaceans by other workers (see Chapter 1, section 1.4.11) have generally involved the use of plasma or serum to challenge bacteria. In the series of experiments described here, the specific intention has been to investigate the antibacterial properties of *C. maenas* haemocytes in isolation from the humoral components of haemolymph.

2.2. Materials and Methods

2.2.1. Animals

Specimens of the common shore crab, *C. maenas*, were collected from St. Andrews Bay in Scotland, kept in a flowing seawater aquarium and fed twice weekly on minced fish. Only healthy male intermoult animals (7-10 cm carapace width) were used for experimental purposes and each animal was subjected to a single bleed. Animals exhibiting marked melanization of the carapace as a consequence of injury, or animals with only a single cheliped, were rejected. Seawater temperature ranged from a maximum of 14 °C to a minimum of 3 °C during the period of experimentation.

2.2.2. Bacteria

Twelve strains of Gram-positive and Gram-negative marine bacteria from geographically diverse waters were used for *in vitro* tests of antibacterial activity in *C. maenas* haemocytes and are listed, together with a description of colony appearance, in Tables 2.1 & 2.2. All were cultured in Bacto Marine Broth 2216 or on Bacto Marine Agar 2216 (Difco Labs).

The three Antarctic isolates, designated BS 66, BS 68 and BS 78, were raised to log phase growth in marine broth at 18 °C for 12-18 h. The temperate water bacteria - *Psychrobacter immobilis, Pseudomonas* 3-1-1, *Photobacterium phosphoreum, Planococcus citreus,* two serotypes of *Vibrio anguillarum* and an avirulent strain of *Aerococcus viridans* (var.) *homari* (= *Gaffkya homari*) - were raised at 20 °C for 24 hours. *Pseudomonas* 1-1-1 (an agar-digester) was cultured on marine agar slopes for 2-3 days at 20 °C and harvested by washing the slopes with sterile 3.2 % NaCl (pH 7.0). Culture of the lobster pathogen *A. viridans* (virulent) was facilitated by supplementing marine broth with lobster serum (1 ml per 50 ml broth). Essentially, serum was prepared using the method described for the shore crab by Smith &

Table 2.1. Gram-positive bacteria used in assays for antibacterial activity

Bacteria	Incubation temp for marine agar plates (°C)	Colony appearance
Planococcus citreus (NCIMB[1] 1493)	32	orange
Aerococcus viridans (var.) *homari* [2] virulent	20	white
Aerococcus viridans (var.) *homari* [2] avirulent	20	white
BS 68[3]	15	buff
BS 66[3]	15	yellow

[1]NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

[2]*Aerococcus viridans* (var.) *homari* from the Department of Agriculture and Fisheries. The virulent strain had been passaged through lobsters and the avirulent strain had been maintained on agar slopes.

[3]Marine Antarctic isolates from surface waters in Bransfield Strait, provided by The British Antarctic Survey.

Table 2.2. Gram-negative bacteria used in assays for antibacterial activity

Bacteria	Incubation temp for marine agar plates (°C)[5]	Colony appearance
Psychrobacter immobilis (NCIMB[1] 308)	32	cream
Photobacterium phosphoreum (NCIMB[1] 844)	20	white, luminescent
Pseudomonas 3-1-1[2]	32	pink
Pseudomonas 1-1-1[2]	20	black, agar digesting
Vibrio anguillarum 8575 (Serotype I)[3]	20	orange-yellow
Vibrio anguillarum 8587 (Serotype II)[3]	20	orange-yellow
BS 78[4]	15	cream

[1]NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

[2]*Pseudomonas* 3-1-1 and *Pseudomonas* 1-1-1 are marine isolates from the west coast of Scotland.

[3]*Vibrio anguillarum* provided by the Institute of Aquaculture, University of Stirling, Scotland.

[4]Marine Antarctic isolates from surface waters in Bransfield Strait, provided by The British Antarctic Survey.

[5]Temperate water bacteria were incubated for 48-72 hours; Antarctic strains for 3-5 days.

Ratcliffe (1978). No serum supplement was used when raising *A. viridans* (virulent or avirulent) on marine agar plates.

Bacteria were prepared by centrifugation at 1,900 g for 10 minutes, washed twice in sterile 3.2 % NaCl (pH 7.0), and finally resuspended in sterile 3.2 % NaCl. Prior to use, bacteria were calibrated by making ten-fold serial dilutions of suspensions prepared to an absorbance of 0.5 at 570 nm. One hundred microlitre aliquots of each dilution were spread on marine agar plates using a sterile glass spreader. Plates were incubated at the appropriate temperature (Tables 2.1 & 2.2) and the dilutions giving between 50-100 colonies per plate were used to calculate the concentration of the original suspensions.

Thereafter, for experiments, all bacteria were routinely standardized to an absorbance of 0.5 at 570 nm and serially diluted in sterile 3.2 % NaCl to give concentrations of between 2×10^6 and 5×10^6 bacteria ml^{-1} before use in antibacterial assays.

A sterile technique was employed for all the antibacterial assays described in this thesis. In addition, glassware used in the preparation of samples was rendered pyrogen free by baking for 1 h at 160 °C. All chemicals, unless otherwise indicated, were from BDH, UK. Recipes for buffers are set out in Appendix I.

2.2.3. Buffer selection

Preliminary control growth experiments were carried out to select an appropriate buffer for antibacterial assays and for the preparation of haemocyte lysate supernatants (HLS). In the work by Söderhäll & Smith (1983) which examined phenoloxidase activity in the shore crab, HLS was optimally prepared in a 0.01 M sodium cacodylate buffer. However, cacodylate has known cytotoxic properties and was likely to be unsuitable for supporting bacterial growth.

Viability tests were carried out by incubating a known concentration of bacteria in a mixture of marine broth plus one of the following: complete *Carcinus* saline (CS I), pH 7.4 (Smith & Ratcliffe 1978); *Carcinus* saline modified to contain 20 mM Ca^{++} and no Mg^{++} (CS II), pH 7.4; cacodylate buffer (CAC), pH 7.0; filtered sea water or 3.2 % NaCl (pH 7.0). One Gram-positive and one Gram-negative bacterium were tested (BS 68 and *P.* 1-1-1 respectively) and an incubation time of 6 h was used. Growth assays were carried out twice and the protocol followed that described in detail below for the control tubes in the antibacterial assay.

It was important that the selected buffer supported a stable phenoloxidase enzyme in the HLS preparations, since the relationship between this and antibacterial activity was to be examined at a later stage (see Chapter 4). Thus, the buffer that was finally chosen for antibacterial activity assays was also used to prepare an HLS which was then tested for phenoloxidase activity as described in section 2.2.6.

2.2.4. Preparation of haemocyte lysate supernatant (HLS)

Haemolymph in sterile marine anticoagulant (MAC), pH 4.6 (see Appendix I), was obtained aseptically via the unsclerotized part of a cheliped (Figure 2.1), as described in Söderhäll and Smith (1983), using 5-7 crabs for each HLS preparation. Samples were pooled and centrifuged at 1,900 g (10 min at 4 °C). The resultant haemocyte pellet was washed once in sterile CAC buffer, pH 7.0 and centrifugation was repeated. As a result of the experiments described in section 2.2.3, modified *Carcinus* saline (CS II), pH 7.4, had emerged as the most appropriate buffer for HLS preparation. Thus the cell pellet was homogenized for 10 min in 5 ml sterile CS II, using an ice-cold, pyrogen-free, glass piston homogenizer, and was finally centrifuged in a sterile polycarbonate tube at 40,000 g (20 min at 4 °C). The supernatant, designated HLS, was stored on ice for no more than 15 min before use

Figure 2.1

Figure 2.1. Removal of haemolymph from *Carcinus maenas* via the unsclerotized part of a cheliped. Haemolymph (2 ml) was withdrawn into a sterile 5 ml syringe containing 2.5 ml marine anticoagulant (MAC) as described in Materials and Methods, page 60.

in antibacterial assays. Ice-cold buffers were used during the preparation of all cell lysate supernatants.

2.2.5. Assay for antibacterial activity in HLS

The standard assay involved incubation of 900 μl of HLS with 100 μl of bacterial suspension in sterile Eppendorf tubes to give a final concentration of 2-5 x 10^5 bacteria ml^{-1}. Controls comprised equal parts of sterile marine broth and sterile CS II as a substitute for HLS, and experimental and control tubes were incubated at 20 °C. At appropriate intervals, as required, 100 μl aliquots of test or control solutions were removed and serially diluted (1:10 in sterile 3.2 % NaCl, pH 7.0) to give countable colonies. One hundred microlitres of the last two dilutions were plated out in triplicate on marine agar and plates were incubated for 48-72 hours at the temperatures indicated in Table 2.1 & 2.2. Colony forming units were recorded for the sets of three plates that gave the most satisfactory counts (usually between 50 and 150 colonies) and the median figure of each set was used to calculate antibacterial activity at each time point.

Antibacterial activity was recorded as a survival index (SI) as given in Wardlaw and Unkles (1978) :

$$SI = \frac{\text{Number of colonies at time t}}{\text{Number of colonies at time 0}} \times 100$$

Hence, an SI greater than 100 indicates growth and one of less than 100 indicates antibacterial activity.

2.2.6. Phenoloxidase assay

Phenoloxidase was measured following the protocol described in Söderhäll & Smith (1983). Briefly, 100 μl HLS was preactivated with 100 μl elicitor (lipopolysaccharide (LPS) from *E. coli* 0111: B4, phenolic extraction, Sigma. Poole,

Dorset) (1 mg ml^{-1} in CS II) for 20 min at 20 °C prior to addition of 100 μl of the substrate L-dihydroxyphenylalanine (L-dopa) (3 mg ml^{-1}). The reaction was timed and finally terminated by the addition of 300 μl of CS II and absorbance was read at 490 nm. Modified *Carcinus* saline (CS II) was substituted for HLS in the buffer controls. Enzyme activity is expressed as units min^{-1} mg $protein^{-1}$. One unit is the amount of enzyme activity which gives an increase in absorbance of 0.001 at 490 nm at 20 °C under the experimental conditions.

2.2.7. Optimal *in vitro* temperature for antibacterial assays

The antibacterial assays described above were performed at 20 °C, largely for convenience. However, this was not necessarily the optimal temperature for *in vitro* antibacterial activity. Therefore, experiments were carried out at a number of incubation points between 0 °C and 20 °C to optimize the *in vitro* system before proceeding with further work.

Experimental and control tubes were set up in the usual way (section 2.2.5) with *P. immobilis* as the test organism. Experiments were carried out as paired comparisons at selected incubation temperatures (i.e, between 20 °C and 15 °C, 10 °C, 5 °C, and 0 °C). After 4 h, aliquots were removed, serially diluted and plated out on marine agar. Each experiment was repeated three times and SI values were calculated for the individual incubation temperatures. Data were analysed by paired or independent t-tests as appropriate.

2.2.8. Protein determination

Protein was measured for each HLS using the method described by Bradford (1976). Bovine serum albumin (BSA) was used as standard. Protein concentrations for HLS were between 1.5 and 1.8 mg ml^{-1} for spectrum of activity experiments and between 1.2 and 1.5 mg ml^{-1} for experiments relating to optimal *in vitro* temperature.

2.3. Results

2.3.1. Buffer selection

Tables 2.3 and 2.4 show the results of incubating BS 68 and *Pseudomonas* 1-1-1 in the five different media given in Materials and Methods. Data in Table 2.3 and 2.4 are mean values for two repeat experiments. Survival indices (SI) were recorded at 3 h and 6 h. Tables 2.3 and 2.4 show that for both BS 68 and *P.* 1-1-1, CS II gave the best growth over this time interval, with SI values in excess of 250. Sodium chloride (3.2%), CS I and filtered sea water also gave acceptable SI values (272 ± 30, 165 ± 32 and 119 ± 15 respectively) for BS 68 (Table 2.3) but of these three media, only seawater encouraged good growth in *P.* 1-1-1, giving an SI of 192 ± 27 after 6 h (Table 2.4). The cacodylate buffer did not support bacterial growth over 6 h (Tables 2.3 & 2.4), giving respective SI values of 75 ± 22 and 98 ± 22 for BS 68 and *P.* 1-1-1. Consequently, it was determined that CS II would be the buffer of choice for the projected work.

2.3.2. Phenoloxidase activity

Phenoloxidase assays were carried out on HLS prepared in CS II, with CS II substituted for HLS in control tubes. Low activity was observed in the control tubes (typically, 10-20 units min^{-1} mg^{-1} protein), but enhanced activity was recorded for the mixtures containing LPS as an elicitor (70-120 units min^{-1} mg^{-1} protein).

2.3.3. Assays for antibacterial activity

In control tubes, all twelve species of bacteria had SI values in excess of 100 after an incubation period of 6 h (Figure 2.2, A-E & Figure 2.3, A-G). Of the five Gram-positive organisms tested, two, BS 66 and BS 68 (the Antarctic bacteria), were particularly sensitive to the antibacterial effect, with SI values approximating to zero

Table 2.3. Effect of buffers on the growth of BS 68

Medium[1]	SI 3h[5]	SI 6h[5]
3.2 % NaCl	154 ± 15	272 ± 30
Complete *Carcinus* saline[2]	150 ± 26	165 ± 32
Modified *Carcinus* saline[3]	160 ± 12	350 ± 28
Cacodylate buffer[4]	45 ± 10	75 ± 22
Seawater	113 ± 11	119 ± 15

[1]Medium: Comprised 450 μl of 3.2 % NaCl (pH 7.0), complete *Carcinus* saline (CS I; pH 7.4), modified *Carcinus* saline (CS II; pH 7.4), cacodylate buffer (CAC; pH 7.0) or filtered seawater, together with 450 μl marine broth as described in Materials and Methods, page 64.

[2]Complete *Carcinus* saline (CS I): prepared as described in Appendix I (from Smith & Ratcliffe, 1978).

[3]Modified *Carcinus* saline (CS II): modified from Smith and Ratcliffe, (1978) (see above), to contain 20 mM $CaCl_2$. $6H_2O$ and no $MgCl_2$. $6H_2O$.

[4]Cacodylate buffer (CAC): prepared as described in Appendix I.

[5]SI: survival index: calculated as described in Materials and Methods, page 63.

Table 2.4. Effect of buffers on the growth of *Pseudomonas* 1-1-1

Medium[1]	SI 3h[5]	SI 6h[5]
3.2 % NaCl	66 ± 13	67 ± 15
Complete *Carcinus* saline[2]	101 ± 20	105 ± 12
Modified *Carcinus* saline[3]	153 ± 27	310 ± 35
Cacodylate buffer[4]	61 ± 14	98 ± 22
Seawater	139 ± 19	192 ± 27

[1]Medium: Comprised 450 µl of 3.2 % NaCl (pH 7.0), complete *Carcinus* saline (CS I; pH 7.4), modified *Carcinus* saline (CS II; pH 7.4), cacodylate buffer (CAC; pH 7.0) or filtered seawater, together with 450 µl marine broth as described in Materials and Methods, page 64.

[2]Complete *Carcinus* saline (CS I): prepared as described in Appendix I (from Smith & Ratcliffe, 1978).

[3]Modified *Carcinus* saline (CS II): modified from Smith and Ratcliffe, (1978) (see above), to contain 20 mM $CaCl_2$. $6H_2O$ and no $MgCl_2$. $6H_2O$.

[4]Cacodylate buffer (CAC): prepared as described in Appendix I.

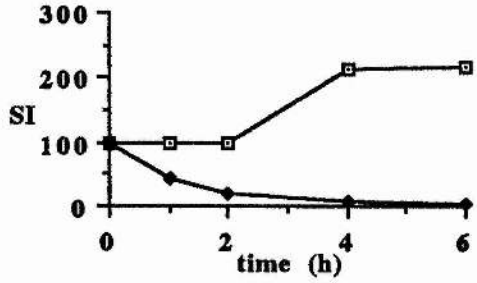
[5]SI: survival index: calculated as described in Materials and Methods, page 63.

Figure 2.2

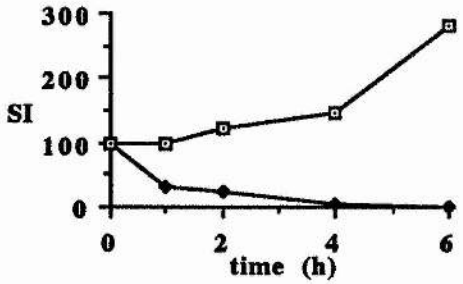
Figure 2.2 Effect of *Carcinus maenas* haemocyte lysate supernatant (HLS) on the survival of Gram-positive bacteria. HLS was prepared as described in Materials and Methods, page 60, and survival indices (SI) were calculated as given in Materials and Methods, page 63. Data relate to individual experiments. Repeat experiments all showed the same trends. Controls comprised equal parts of marine broth and modified *Carcinus* saline (CS II) as a substitute for HLS.

Key: Experimental ♦
Control ¤

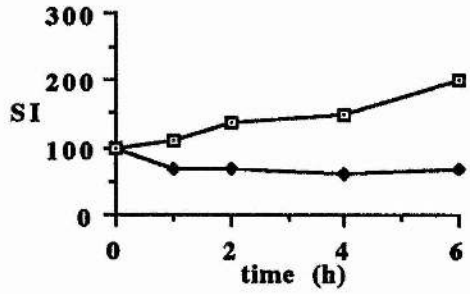
A. BS 66, Antarctic marine strain



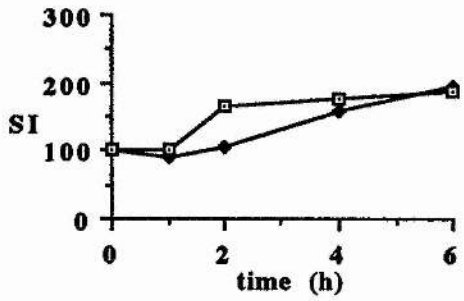
B. BS 68, Antarctic marine strain.



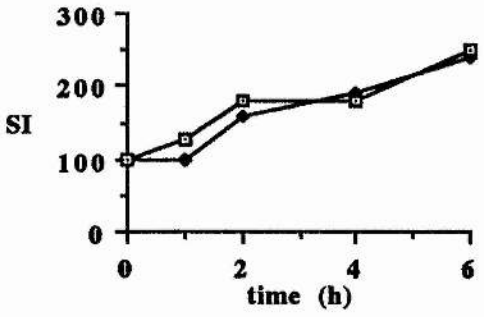
C. *Planococcus citreus*



D. *Aerococcus viridans* (var.) *homari* (virulent)



E. *Aerococcus viridans* (var.) *homari* (avirulent)



after 6 h (Figure 2.2A, B). These organisms also responded rapidly within the first hour of challenge, as indicated by the slope of the curves in Figure 2.2A, B. Another Gram positive bacterium, *Planococcus citreus,* also exhibited a reduction in SI (but to 67.4 after 6 h), and compared with BS 66 and BS 68, the response was slower (Figure 2.2C). By contrast, both virulent and avirulent strains of *Aerococcus viridans* flourished in the presence of HLS, with final SI values of 190 and 240 respectively (Figure 2.2D, E), growth patterns closely following those of their respective controls.

Five of the seven Gram-negative bacteria were sensitive to the effect of HLS, but to different degrees. *Pseudomonas* 1-1-1 (Figure 2.3A), *Psychrobacter immobilis* (Figure 2.3B) and BS 78 (Figure 2.3C) produced SI values approaching zero within 4 h, whilst the two serotypes of *Vibrio anguillarum* (Figure 2.3D, E), gave an intermediate result, with survival indices of 57 for serotype I (Figure 2.3D) and 45 for serotype II (Figure 2.3E) after 6 h. Of the two remaining Gram-negative bacteria, *Pseudomonas* 3-1-1 gave a response that was similar to that of the corresponding control with an SI of 112, indicating a small amount of growth over 6 h (Figure 2.3F). On the other hand, *Photobacterium phosphoreum* grew in the presence of HLS over the same time interval (to give an SI of 340), but to a lesser extent than the associated control where the SI was over 830 (Figure 2.3G).

Overall, the antibacterial effect was found to operate against eight of the twelve species challenged with HLS from *C. maenas.* All data presented here relate to individual representative experiments and the same trends were noted in all replicated investigations.

2.3.4. Optimal *in vitro* temperature for antibacterial activity

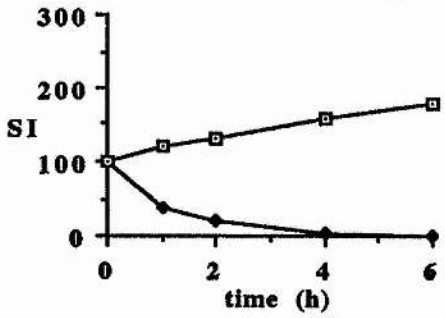
In this set of experiments, 20 °C was found to be the optimal *in vitro* temperature for antibacterial activity in HLS. Paired comparisons of survival indices were made between bacteria incubated with HLS at 20 °C and bacteria incubated with HLS at the

Figure 2.3

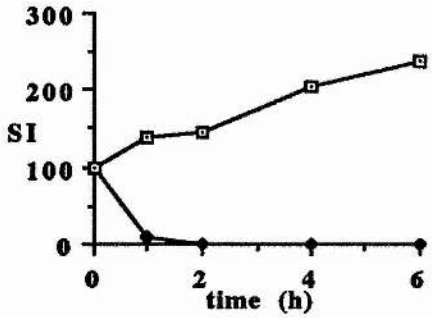
Figure 2. 3. Effect of haemocyte lysate supernatants (HLS) on the survival of Gram-negative bacteria. HLS was prepared as described in Materials and Methods, page 60, and survival indices (SI) were calculated as given in Materials and Methods, page 63 . Data relate to individual experiments. Repeat experiments all showed the same trends. Controls comprised equal parts of marine broth and modified *Carcinus* saline (CS II) as a substitute for HLS

Key: Experimental ♦
Control ¤

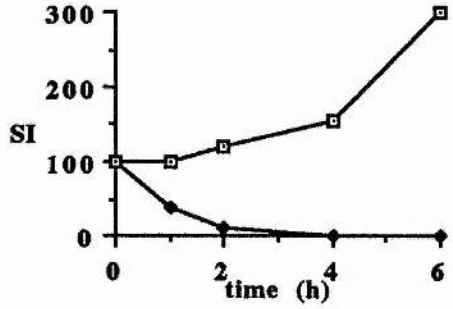
A. *Pseudomonas* 1-1-1



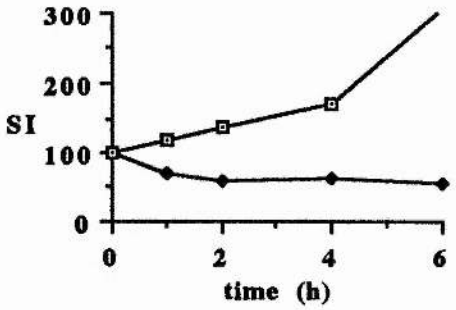
B. *Psychrobacter immobilis*



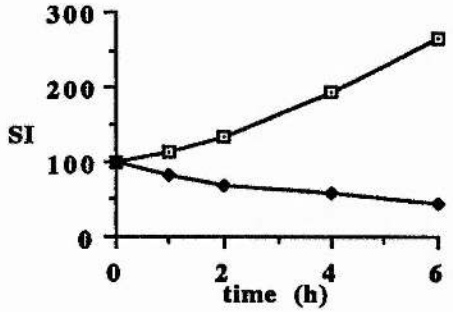
C. BS 78, Marine Antarctic bacterium



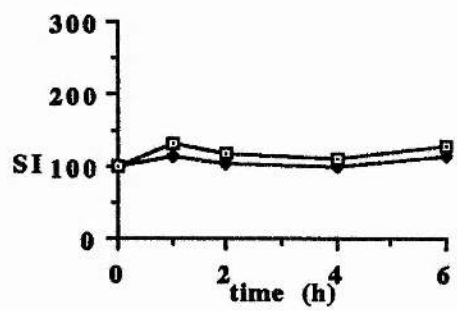
D. *Vibrio anguillarum* (Serotype I)



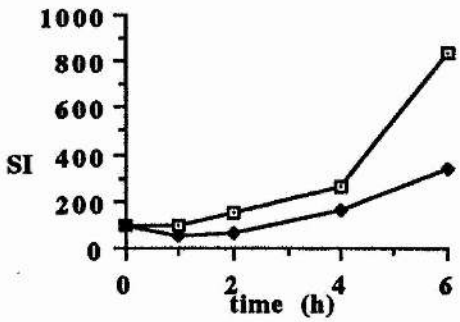
E. *Vibrio anguillarum* (Serotype II)



F. *Pseudomonas* 3-1-1



G. *Photobacterium phosphoreum*



other three temperatures. Table 2.5 gives data from repeat experiments and demonstrates that antibacterial activity in HLS is greatest at an incubation temperature of 20 °C. Paired t-tests gave significant differences between SI values at 20 °C incubation compared with all the other temperatures ($p < 0.05$ in all cases). Nevertheless, antibacterial activity was present in HLS at all temperatures (see Table 2.5), and independent t-tests used to cross test for significance between treatments showed no significant difference between SI values for 0 °C and 10 °C ($t = -2.2290$, $p = 0.09$) or 5 °C and 10 °C ($t = 2.4495$, $p = 0.07$). All other comparisons were significant with $p < 0.01$. In all experiments, bacteria in controls showed growth (Table 2.5).

2.4. Discussion

This chapter has examined the antibacterial activity of *C. maenas* HLS using twelve marine bacterial species, including two known pathogens. An essential part of developing the assay was the selection of a suitable buffer system that satisfied two requirements: firstly the buffer had to support *in vitro* bacterial growth over the allotted time interval and secondly it was desirable that HLS preparations have a stable proPO system. Although bacterial growth was not exclusive to modified *Carcinus* saline (CS II), overall it gave the best results and avoided the difficulties inherent in using seawater - namely, the unknown variations in composition which may arise, thus introducing uncontrollable variables into the experimental work. In addition, phenoloxidase activity could be elicited in HLS that had been prepared in CS II, and showed approximately 7-10 fold increase in activity above the controls.

The results of the antibacterial assays demonstrate that an antibacterial factor (or factors) resides in the haemocytes, operates against both Gram-positive and Gram-

Table 2.5. Effect of incubation temperature on *in vitro* antibacterial activity in HLS[1] from the shore crab, *Carcinus maenas*

4 h Survival indices at different incubation temperatures[2]								
20 °C (HLS)[3]	0 °C (HLS)	0 °C (Control)	5 °C (HLS)	5 °C (Control)	10 °C (HLS)	10 °C (Control)	15 °C (HLS)	15 °C (Control)
0.1	44.9	120.4						
3.4	40.0	104.5						
0.1	40.3	100.2						
1.5			75.0	108.6				
4.0			64.6	110.5				
3.7			82.7	100.0				
6.5					44.1	109.5		
7.0					63.6	100.7		
4.2					57.4	105.7		
0.1							16.1	118.0
1.0							24.2	126.0
4.6							26.7	136.7

[1]HLS: Haemocyte lysate supernatant was prepared as described in Materials and Methods, page 51.

[2]Values are for individual experiments which were set up as paired comparisons as described in Materials and Methods, page 53.

[3]For the sake of clarity, control values for incubations at 20 °C have been omitted. All controls at this temperature showed growth over four hours with SI values in excess of 100. Controls contained equal parts of modified *Carcinus* saline (CS II) and marine broth as a substitute for HLS.

negative organisms (including those from geographically remote waters) and is rapid in action.

Amongst the Gram-positive strains tested, the strongest antibacterial activity, as evidenced by low survival indices, was against the two Antarctic bacteria (BS 66 & BS 68). This contrasts sharply with the apparant lack of antibacterial effect on both virulent and avirulent strains of *Aerococcus viridans*. Similarly, the SI values of the the Gram-negative bacteria, *Psychrobacter immobilis, Pseudomonas* 1-1-1 and BS 78 were markedly depressed when challenged with HLS, whereas *Photobacterium phosphoreum* and *Pseudomonas* 3-1-1 were not. This variability in response by both Gram-negative and Gram-positive forms suggests that there may be particular characteristics of some organisms which render them more susceptible than others to the antibacterial factor in HLS.

The resistance of both virulent and avirulent strains of *A. viridans* to challenge with HLS preparations is compatible with the mild pathogenicity that this bacterium is known to exhibit towards some species of crab (Cornick & Stewart, 1968b, 1975). It has been shown in lobsters that haemolymph is an excellent nutrient medium for *A. viridans* and, despite being phagocytosed or encapsulated, the bacterium is capable of continued growth *in vivo* (Cornick & Stewart, 1968a,b), to the detriment of the host.

The final SI values of the Antarctic strains in control solutions (all in excess of 200) indicate that, for the duration of the experiment, the incubation temperature of 20 °C was not incompatible with growth. Indeed, many polar bacteria are capable of growth at this temperature (Herbert, 1986).

For further investigations into the characteristics of the antibacterial activity in crab haemocytes, one Gram-negative bacterium, *P. immobilis,* was used as a sentinel organism. *Psychrobacter immobilis* was selected largely because of its ease of culture and sensitivity to the antibacterial activity in HLS.

The main objective of the *in vitro* study of antibacterial activity at different incubation temperatures was to establish optimal conditions for future experiments. It has been shown here that a working temperature of 20 °C elicits the strongest antibacterial activity when compared with other, lower incubation temperatures and is also compatible with the phenoloxidase assays to be used in subsequent work. The reason for the similarity between results at 0 °C and 10 °C, and 5 °C and 10 °C is not known. Precautions were taken to avoid both temperature variation in incubated samples and thermal shock during serial dilution and plating out, but experimental error remains a possibility.

Identification of a cell-bound antibacterial factor gives rise to a number of questions. Firstly, in which haemocyte population does the factor reside? Preliminary work by Söderhäll & Smith (1986b) has indicated that the granular cells carry the antibacterial factor; these same cells also carry the proPO system, a cascade of enzymes which have been implicated in non-self recognition in arthropods (see Chapter 1, page 20 for references). If the granular cell location of the antibacterial factor is confirmed, then the possibility of a relationship between the proPO system and antibacterial function needs to be explored. Another question, which arises as a direct consequence of the work carried out for this chapter, concerns the possibility of seasonal variation of antibacterial activity in the crab haemocytes. Whilst every effort was made to carry out experiments pertaining to individual bacteria within a short space of time, inevitably the entire series of experiments, involving twelve species of bacteria, bridged 2-3 seasons. It was important, therefore, to determine whether there were any significant fluctuations in the effectiveness of antibacterial activity on a month to month basis. Thus, the following chapters will address these and other questions in an effort to characterize this crustacean antibacterial factor more fully.

Chapter 3
Seasonal Variation of Antibacterial Activity in *Carcinus maenas* Haemocytes

3.1. Introduction

Carcinus maenas shows physiological adaptation to a wide range of temperatures and salinities (Mantel 1983), and spends a proportion of its time in the intertidal zone where it usually avoids thermal shock and dehydration throughout the year (Naylor, 1962). Crabs as a group have been well researched with respect to several aspects of their physiology (see Chapter 1, page 45) and in some instances there are reports of associated seasonal variation in the parameters measured (for examples see, Naylor, 1963; Todd, 1963; Naylor *et al.*, 1971; Howell *et al.*, 1973; Chang & O'Connor, 1983).

In common with many other animals, crustaceans are influenced by lunar and solar cycles, and exhibit diurnal and annual biological rhythms with respect to many and varied functions (De Coursey, 1983), so it is surprising to find that seasonal studies are scant with regard to the host defence system. Of those studies that have been carried out, none relate to antibacterial activity of the type under examination in this thesis, although a few authors have recorded variations in humoral activity in response to changes in temperature or season. For example, Dean & Vernberg (1966) have found that temperature affects clotting times, total haemocyte count and levels of plasma protein in the hermit crab, *Uca pugilator*, with cold acclimated animals having slower clotting times and lower protein concentrations than those maintained at higher temperatures. Peters & Long (1973) also found that temperature affected clotting times in the purple shore crab, *Hemigrapsus nudus*, but noted that clotting times were faster and protein levels were higher in the cold acclimated animals, which is in direct contradiction to Dean & Vernberg's results (1966). This may be a reflection of different acclimation patterns in the two animals (Peters & Long, 1973). More recently, Muramoto *et al.*, (1991) have recorded increased agglutinin activity in the acorn barnacle (*Megabalanus rosa*) during the summer months compared with the

winter. Whether this is a direct consequence of temperature or is due to other physiological factors is unclear.

As shown in Chapter 2, antibacterial activity resides in the haemocytes of *C. maenas* and, for each bacterium tested, the same trends (either activity present/absent) were noted in replicated experiments. However, the initial screening was completed over several months and the results may have been influenced by seasonal variation in antibacterial activity. Therefore, seasonality was investigated in order to understand how the animal's defence capability might be affected by environmental factors. With only limited time available, this survey was carried out at monthly intervals for a period of twelve months (February 1991-January 1992).

3.2. Materials and Methods

Experiments were carried out on three consecutive days of each month for a period of one year (February 1991-January 1992), and the ambient temperature of aquarium seawater (± 0.5 °C) was recorded on each experimental day. Using the protocol described previously in Chapter 2, page 60, haemocyte lysate supernatant (HLS) samples were prepared and assayed for antibacterial activity against *Psychrobacter immobilis*. The data collected comprised:

a) bacterial survival indices (SI) (see Chapter 2, page 63)

b) HLS protein concentrations (see Chapter 2, page 64)

c) haemocyte counts for each crab (cells ml^{-1} haemolymph)

d) seawater temperature (°C)

Five crabs only were used for each HLS and no attempt was made to standardize the protein levels between samples. Haemocyte counts were carried out on diluted haemolymph samples (usually 2 ml haemolymph in 2.5 ml marine anticoagulant (MAC) using New Improved Neubauer haemocytometers. Data were analysed using Student's t-test for independent samples and also one way ANOVA as appropriate.

3.3. Results

The seasonal study examined *in vitro* antibacterial activity of *C. maenas* HLS in relation to HLS protein concentration, haemocyte counts and seawater temperature at intervals over a period of twelve months. Full data for all repeat experiments over the twelve month period are shown in Appendix IIa. Mean values for all data, with standard deviations, are shown in Appendix IIb.

All the parameters measured over 12 months gave a wide range of values (Appendix IIa). Haemocyte counts in individual crabs ranged from a maximum of 7.76×10^7 ml^{-1} in May to a minimum of 0.39×10^7 ml^{-1} in August, while HLS protein ranged from a maximum of 2.0 mg ml^{-1} in June to a minimum of 0.5 mg ml^{-1} in August. Water temperature was maximal in August at 16.5 °C and minimal in February at 3 °C. Survival indices recorded for antibacterial assays ranged between 35.8 for February and, for several months of the year, 0.001 (January, June, July, September, November and December) (Appendix IIa). Antibacterial activity was strong for most of the year, but showed two distinct months (February and August) when activity was less efficient and SI values were elevated (Appendix IIb).

3.3.1. Temperature effects

The mean water temperatures recorded in August and February were significantly lower (February: t-tests: $p < 0.0001$) and higher (August: t tests: $p = 0.002$) than temperatures recorded in other months (see Appendix IIb for mean seawater temperatures).

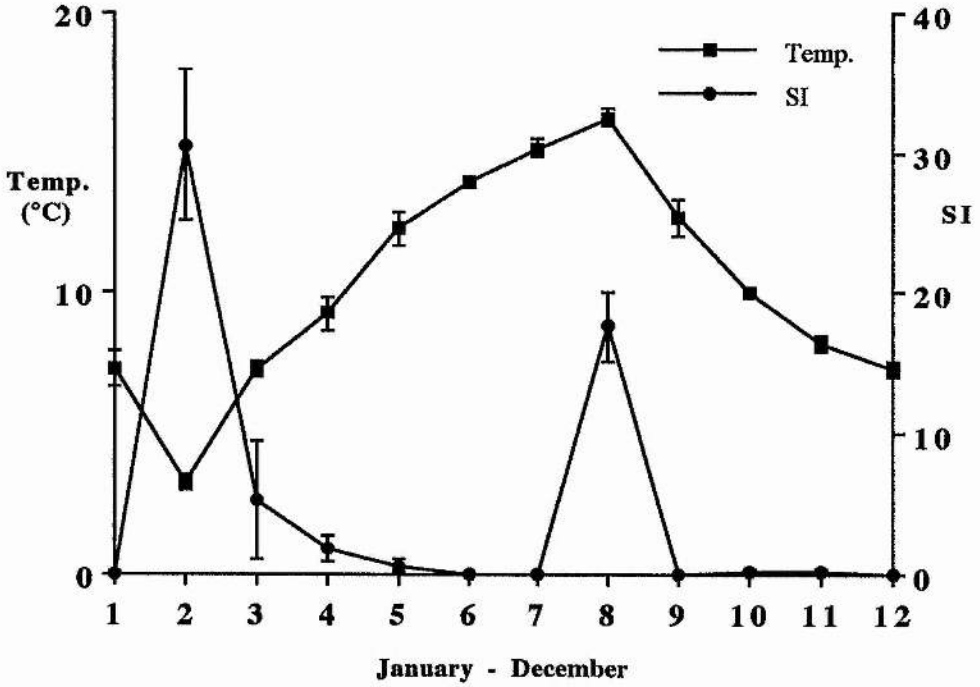
Evaluation of antibacterial activity in relation to seawater temperature by one way ANOVA gave a highly significant temperature effect ($F = 43.71$ and $p = 0.001$; $F_{0.01\ (14,\ 21)} < 4.43$). However, February and August data also showed the upper and lower extremes of temperature for the twelve months (3.3 ± 0.3 °C and 16.3 ± 0.3 °C respectively; Appendix IIb) and re-evaluation of the data, excluding that of February and August, showed no influence of temperature on antibacterial activity for the remaining months of the year (one way ANOVA: $F = 0.73$ and $p = 0.677$: $F_{0.05\ (10,19)} > 2.35$). Figure 3.1 demonstrates clearly that high SI values (i.e. reduced antibacterial activities) are coincident with with the extremes of temperature recorded for August and February. Between February and August, increase in water temperature is associated with marked decrease in SI (i.e., an increase in antibacterial activity).

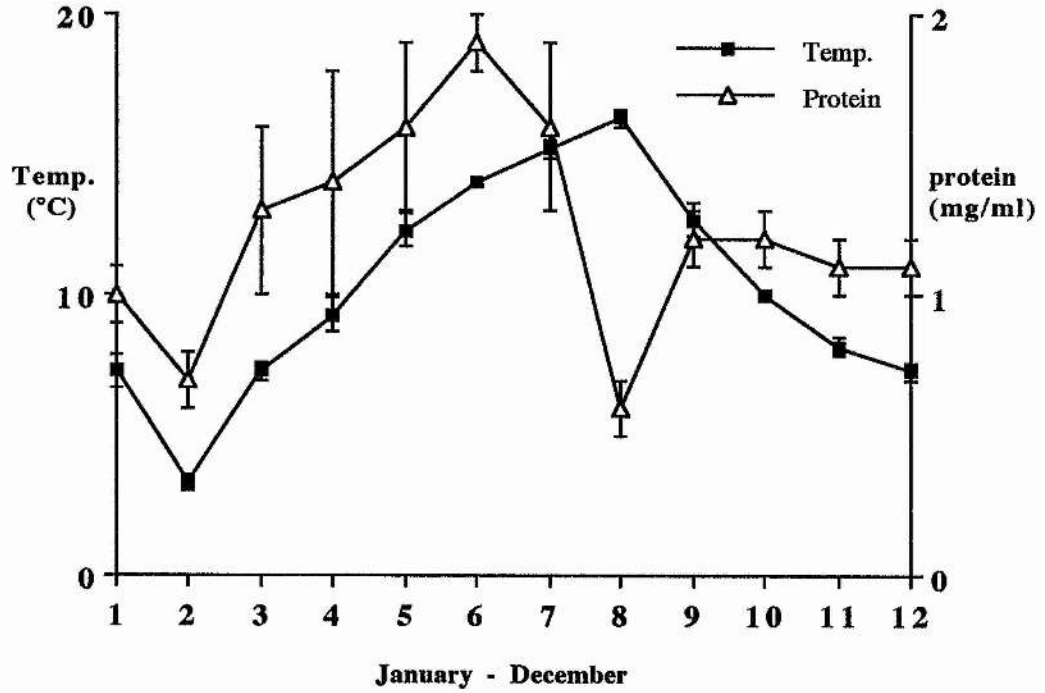
Protein concentrations in HLS were also influenced by changes in water temperature and again this was most apparent at two key points in the year. A marked coincidence of extremes of water temperature and reduced protein levels occured in February and August as shown in Figure 3.2. Analysis of variance revealed a significant temperature effect on HLS protein levels for the year studied (one way ANOVA: $F = 7.50$ and $p < 0.001$; $F_{0.01\ (14,21)} < 2.89$), but this was due largely to the close association between low protein and extreme water temperatures for two particular months. Exclusion of February and August data from the ANOVA showed that protein levels for remaining months were not significantly affected by temperature (one way ANOVA: $F = 2.18$ and $p = 0.073$: $F_{0.05\ (9,19)} = 3.29$), although Figure 3.2

Figures 3.1 & 3.2

Figure 3.1. Survival indices (SI) for *Psychrobacter immobilis,* and mean monthly water temperature. Survival index calculated as given in Materials and Methods, Chapter 2, page 63. High SI values indicate low antibacterial activity. Water temperature (°C) was measured on three consecutive days of each month. Mean Control values are given in Appendix IIb and are all in excess of 100, indicating growth. Values shown are means ± standard deviations (n=3).

Figure 3.2. Mean protein levels in haemocyte lysate supernatants (HLS) and mean water temperature. Haemocyte lysate supernatants were prepared from five animals as described in Materials and Methods, Chapter 2, page 60. Protein was measured as described in Materials and Methods, Chapter 2, page 64. Water temperature (°C) was measured on three consecutive days of each month. Values shown are means ± standard deviations.


20
10
0
Temp.
(°C)
40
30
20
10
0
SI
Temp.
SI
1
2
3
4
5
6
7
8
9
10
11
12
January - December


20
10
0
Temp.
(°C)
2
1
0
protein
(mg/ml)
Temp.
Protein
1
2
3
4
5
6
7
8
9
10
11
12
January - December

suggests that between February and June increases in water temperature are parallelled by increases in protein concentration.

The t-statistic and p value for month-month comparisons of HLS protein concentrations are listed in Appendix IIc. Haemocyte lysate supernatants from February and August contained significantly less protein compared with HLS from other months (t-test: $p < 0.05$ in all cases: Appendix IIc) but February and August values were not significantly different from each other.

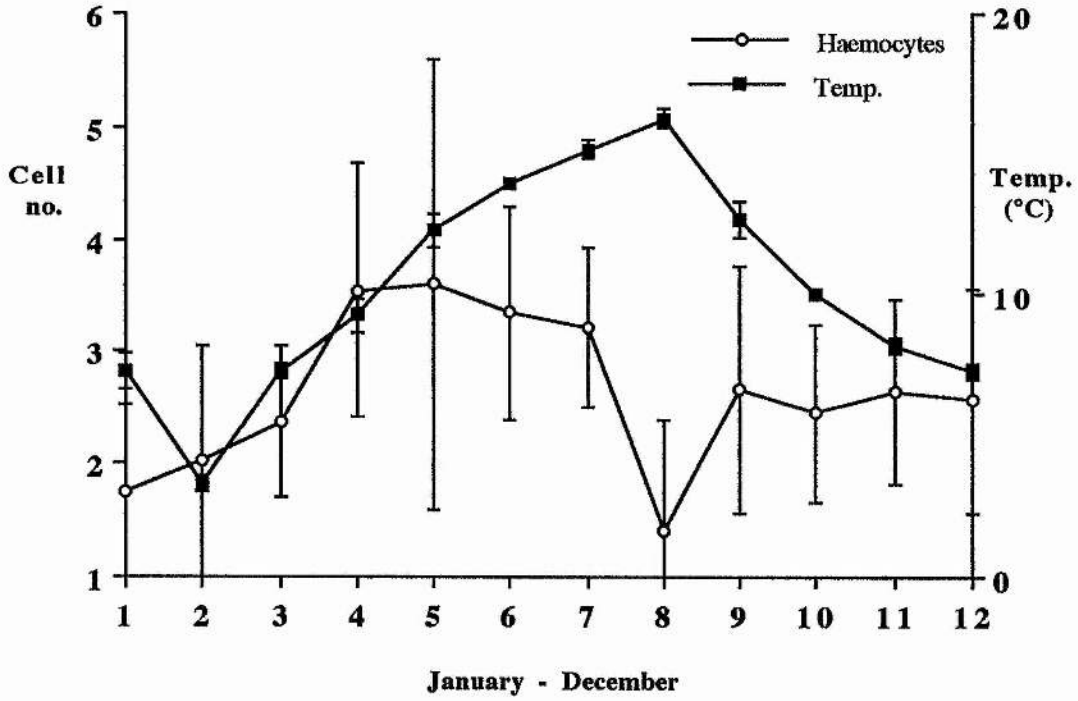
Mean haemocyte counts for batches of five crabs (Appendix IIb) showed significant variation with respect to seawater temperature (one way ANOVA: $F = 8.27$ and $p < 0.01$; $F_{0.01\ (12,\infty)} = 2.18$) and even when data relating to the extremes of temperature (3 °C, 3.5 °C, 16 °C and 16.5 °C) were excluded from the analysis, a highly significant temperature effect remained (one way ANOVA $F = 6.0136$, $p < 0.001$; $F_{0.01\ (8,\infty)} = 2.51$). However, there appears to be no clear trend in the relationship between water temperature and haemocyte count from month to month (Figure 3.3), although in August, a month of extreme high water temperature, crabs showed the lowest mean haemocyte count, compared with all months apart from January and February (Figure 3.3). Appendix IId gives the p values and t-statistic for comparisons of haemocyte counts between months.

3.3.2. Antibacterial activity

Haemocyte lysate supernatants showed powerful antibacterial activity for all months with the exception of February (mean SI 30.6 ± 5.4; n=3) and August (mean SI 17.7 ± 2.5 n=3). Nevertheless, these latter values still represent, *in vitro*, a potent antimicrobial effect. Significant monthly variation in antibacterial activity was found when data were analysed by one way ANOVA ($F = 61.17$ and $p < 0.001$; $F_{0,01\ (11,\ 24)} = 3.09$) but examination of means and standard deviations indicates that the SI values for February and August (see Appendix IIb) have strongly influenced this

Figure 3.3

Figure 3.3. Mean monthly haemocyte counts (x 10^7) in relation to mean water temperature. Haemocyte counts are means ± standard deviations for the total of fifteen crabs used in three repeat experiments in each month. Water temperature (°C) was measured on three consecutive days of each month. Values are means ± standard deviations (n=3).


Haemocytes
Temp.
Cell no.
Temp. (°C)
6
5
4
3
2
1
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
January - December

result. In addition, the standard deviations for January and December exceed the respective mean values and are not significant statistically (Appendix IIb).

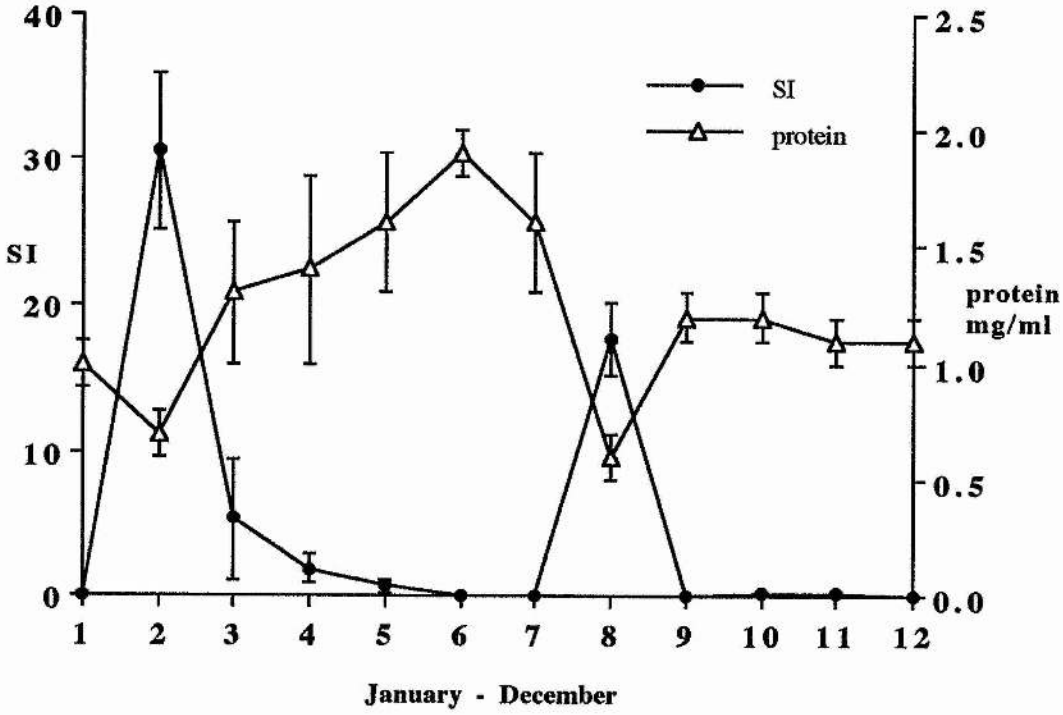
The t-test statistics and associated p values for month - month comparisons of mean SI values (excluding data for January and December) are shown in Appendix IIe. Independent t - tests showed that mean SI values for February and August were not significantly different from each other ($t = -0.3536$; $p = 0.742$), but both were significantly different from those recorded in all other months tested ($p > 0.05$ for all cases). For many data points, standard deviations are large (Appendix IIb), which is a reflection of the small sample sizes.

Antibacterial activity was only significantly influenced by protein levels if February and August data were included; adjusted data gave one way ANOVA: $F = 1.90$ and $p = 0.114$; $F_{0.05\,(9,19)} = 3.29$. Figure 3.4 shows the mean monthly protein levels and antibacterial activity in HLS samples, and it can be seen that low protein concentration is associated with elevated SI (i.e., reduced antibacterial activity) for August and February. By contrast, higher protein levels are always associated with strong antibacterial activity (Figure 3.4).

Assessment of the variation of haemocyte count with antibacterial activity was complicated by the number of widely differing values for the latter. To overcome this, SI values were grouped into four classes, each reflecting a tenfold difference in antibacterial activity (i.e. values below 0.1, values $\geq 0.1 < 1.0$, values $\geq 1.0 < 10$ and values $\geq 10 < 100$). One way ANOVA showed that, overall, haemocyte count varied significantly with antibacterial activity ($F = 9.72$ and $p = < 0.001$; $F_{0.01\,(3,\infty)} = 3.32$) and when February and August data were excluded from the analysis, there was still a significant effect at the 2.5 % level (one way ANOVA: $F = 3.87$, $p = 0.23$; $F_{0.025\,(2,\infty)} = 3.69$). However, from month to month there appears to be no clear relationship between antibacterial activity and haemocyte counts, mainly because of

Figure 3.4

Figure 3.4. Mean monthly protein levels in haemocyte lysate supernatants (HLS) and survival indices (SI) for *Psychrobacter immobilis*. HLS was prepared as described in Materials and Methods, Chapter 2, page 60. Protein was measured as described in Materials and Methods, Chapter 2, page 64. Survival index (SI) was calculated as given in Materials and Methods, Chapter 2, page 63. High SI values indicate reduced antibacterial activity. Control SI values are given in Appendix IIb and are all in excess of 100, indicating growth. Values are means ± standard deviations.


40
30
20
10
0
SI
2.5
2.0
1.5
1.0
0.5
0.0
protein
mg/ml
SI
protein
1
2
3
4
5
6
7
8
9
10
11
12
January - December

the large standard deviations associated with the latter (Figure 3.5). As might be expected, haemocyte counts also varied significantly with protein levels (one way ANOVA: $F = 6.45$ and $p < 0.01$ for $F_{0.01\ (9,\infty)} = 2.41$) and, in spite of the relatively large standard deviations associated with haemocyte counts, Figure 3.6 suggests that protein level fluctuates in parallel with haemocyte count.

In summary, HLS from February crabs gave the weakest antibacterial activity and this was associated with the lowest water temperatures for the year. August crabs also demonstrated reduced antibacterial activity (although not to the same extent as February animals), which was associated with the highest temperature of the year. Powerful antibacterial activity tended to be associated with high protein concentration which, in turn, was associated with high haemocyte counts. Statistical analysis showed that haemocyte count varied significantly with antibacterial activity, although monthly trends were not clearly identifiable in this regard.

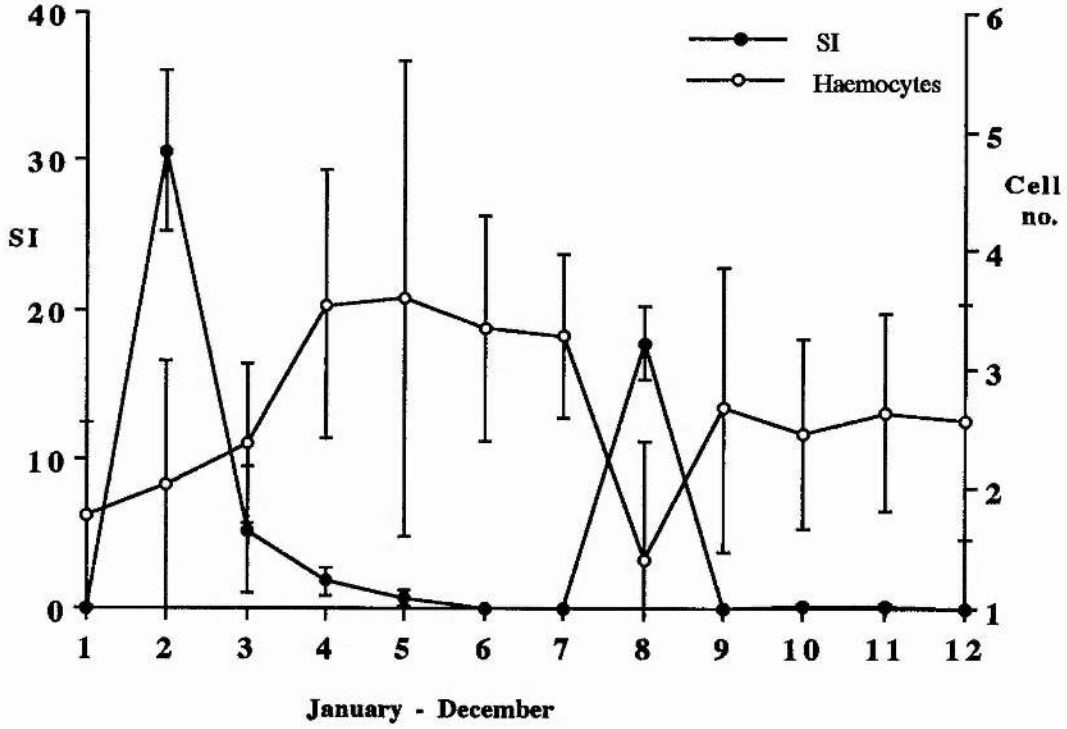
3.3. Discussion

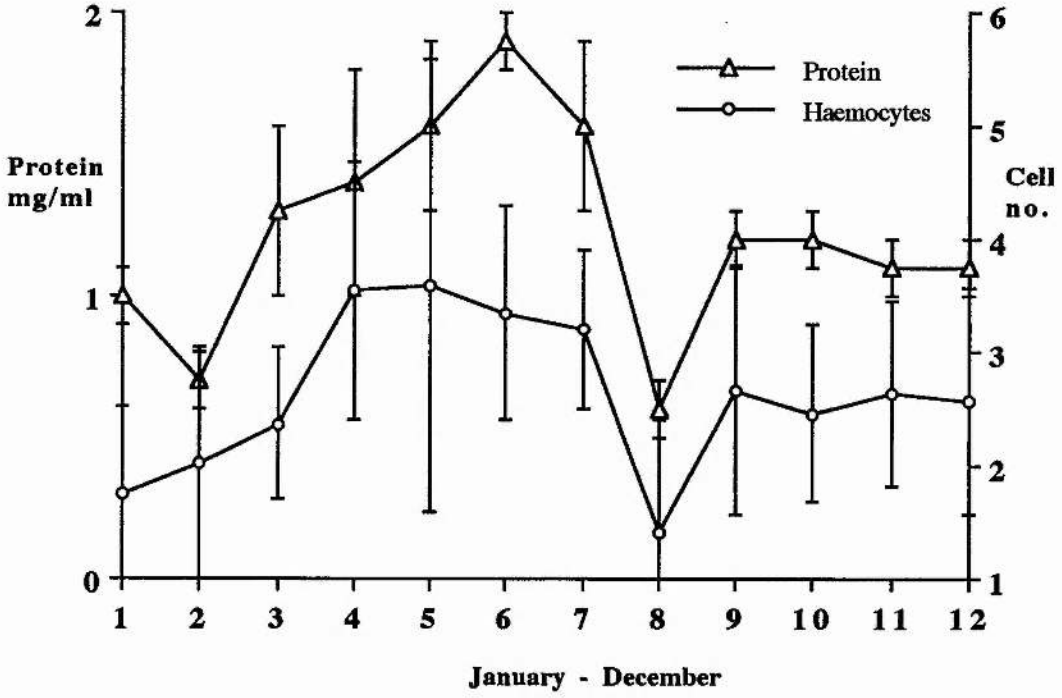
The data presented here shows that antibacterial activity in the haemocytes of the shore crab is influenced by changes in seawater temperature, particularly when temperatures are at the extremes for the year. The two occasions when marked differences in antibacterial activity occurred were in February and August, and occurred in conjunction with water temperatures of 3 °C-3.5 °C and 16 °C-16.5 °C respectively. For other months, despite a wide range of varying temperatures throughout the year, there was very efficient antimicrobial activity (*circa* 90-100 % effective) which was not generally influenced by temperature - for example in January, when water temperature averaged 7.3 °C, and in June and July, when water temperatures were higher (14 °C and 15 °C respectively) antibacterial activity was strong, with no significant differences between the values.

Figure 3.5 & 3.6

Figure 3.5. Mean monthly haemocyte counts ($x10^7$) for crabs and survival indices (SI) for *Psychrobacter immobilis*. Haemocyte counts are means ± standard deviations for the total of fifteen crabs used in each month. Survival index was calculated as given in Materials and Methods, Chapter 2, page 63. Control SI values are given in Appendix IIb and are all in excess of 100, indicating growth. Values are means ± standard deviations (n=3).

Figure 3.6. Mean monthly haemocyte counts ($x10^7$) for crabs, and mean protein levels in haemocyte lysate supernatants (HLS). Haemocyte counts are means ± standard deviations for the total of fifteen crabs used in each month. HLS: Haemocyte lysate supernatants were prepared as described in Materials and Methods, Chapter 2, page 60. Protein was measured as described in Materials and Methods, Chapter 2, page 64.


SI
Haemocytes
SI
Cell no.
40
30
20
10
0
6
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12
January - December


Protein
Haemocytes
Protein mg/ml
Cell no.
2
1
0
6
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12
January - December

A number of points arise from this study. Firstly it is clear that the experimental design would be improved by the incorporation of larger sample sizes in respect of assays for antibacterial activity and protein levels. Secondly, whether the observed effect can be attributed entirely to temperature variation is not known. There may be other environmental and / or physiological factors which exert an influence at these times of year and which may contribute in part to a reduced antibacterial response, either directly or via reduced haemocye counts. In addition, it is arguable that for a phenomenon as powerful as this, the subtle differences between SI values of, for example, 0.1 and 0.01 (representing an effectiveness of 99.90 % and 99.99 % respectively) are perhaps less important than the clear differences seen in February and August.

If temperature directly affects antibacterial activity, then one explanation could be that the antibacterial factor(s) is / are only sensitive to extremes of environmental temperature. As mentioned previously in the introduction to this chapter, *C. maenas* shows general physiological adaptation to a wide range of environmental conditions, and elements of the immune system may be similarly adapted. It is imprudent to use *in vitro* results as a basis for predicting *in vivo* responses; nevertheless it is worth noting that results in Chapter 2 showed that increasing incubation temperature up to 20 °C *in vitro* enhanced rather than depressed antibacterial activity.

An alternative, and more probable, explanation could be that the observations reported here are due to underlying temperature effects on haemocyte counts and protein levels in the blood. Protein, haemocyte counts and water temperature do appear to be inter-linked, as shown by the results in section 3.2, which gives some support to this idea. If this is so, then haemocyte number or protein concentration may well be more important than temperature in determining the level of antibacterial activity in crab blood. Also, since the antibacterial activity reported here is cell based,

then activity must be related, at least in part, to either total cell counts or differential cell counts in individual animals.

Antibacterial activity in February is weaker than in August (SI values were *circa* 30 and 17 respectively), although cell counts and protein levels are lower in August. The low temperature of February (3 °C) could have a greater effect on haemocyte counts and protein than the high temperature of August (16.5 °C): in February, crabs are subjected to diminished food supply and have reduced metabolic activity (Chang & O'Connor, 1983), whereas in August, plentiful food and a general elevation of metabolic rate may to some extent compensate for the adverse effects of temperature.

Temperature-related metabolic responses have been reported for a number of physiological parameters in crustaceans (Mantel, 1983). For the immune system in particular, it has been demonstrated that phagocytosis is temperature dependent (McKay & Jenkin, 1970b; Paterson & Stewart, 1974) and, as previously mentioned on page 78, Dean & Vernberg (1966) and Peters & Long (1973) have reported temperature effects on haemolymph clotting time and protein concentration for *Uca pugilator* and *Hemigrapsus nudus*. It has also been shown that temperature acclimation affects the resistance of the crayfish *Parachaeraps bicarinatus* to infection with a bacterial pathogen following immunisation (McKay & Jenkin, 1969; McKay *et al.*, 1973). Data produced in our laboratory by Karen Bell (cited in Smith & Chisholm, 1992) suggest that acclimation temperature has a powerful effect on haemocyte number and also on *in vitro* antibacterial activity in *C. maenas*. In accordance with similar observations by Dean & Vernberg (1966) for *U. pugilator*, and McKay & Jenkin (1969) for *P. bicarinatus*, the antimicrobial effect of *C. maenas* haemolymph on *Pseudomonas* 1-1-1 is lower in animals acclimated to 19 °C or 13 °C compared with controls maintained at 6 °C (cited in Smith & Chisholm, 1992). Concomitant with this is a reduction in haemocyte count and phenoloxidase activity (Bell, cited in Smith & Chisholm, 1992).

Close association between protein levels and haemocyte counts is not entirely surprising in a haemocyte lysate supernatant. The absence of strong, related trends between antibacterial activity and total haemocyte count may be due a variation in the quality rather than the quantity of protein being synthesised at different times of the year. Also, variation in differential cell counts may occur in individual crabs, which, whilst not necessarily affecting total cell count, may give rise to reduced numbers of cells carrying the antibacterial factor(s). There is the additional possibility that the antimicrobial effect may not be entirely protein-related. Numerous antibacterial substances have been isolated in a diverse collection of invertebrates, (see review by Ratcliffe *et al.*, 1985). Frequently, these antimicrobial molecules are proteins. However, this is not exclusively so; for example, it has been proposed for tunicates that secretion of accumulated vanadium from the vanadocytes in the blood has antimicrobial properties (White & Rowley, 1982) and, at the other end of the phylogenetic spectrum, marine sponges have potent antibacterial and molluscicidal sesterterpenes (Cariello, *et al.*, 1982; König, *et al.*, 1992).

It is important to bear in mind that even robust species of crab, such as *C. maenas,* may encounter unquantifiable stresses in the aquarium environment, quite apart from any natural seasonal elevations or depressions in water temperature. It would be difficult to assess the extent to which extremes of seawater temperature together with artificial environmental conditions might compromise the host immune response at particular times of the year.

There have been a few reports relating to the effects of seasonally-induced stresses on the crustacean immune system. One such study, carried out on stressed and unstressed populations of *Callinectes sapidus* (Welsh & Sizemore, 1985), suggests that seasonal effects may be important in host defence. These authors noted that there were seasonal differences in the bacterial levels in haemolymph from crabs collected by hand and by crab pot, with mean monthly levels of bacteria in haemolymph

increasing with higher mean monthly water temperature. Overall, Welsh & Sizemore (1985) proposed that physiological stresses may facilitate an increase in bacterial numbers by reducing the effectiveness of host defence reactions. These observations could be reinterpreted in the light of the results presented in this chapter - i.e., that reduced antibacterial activity is found in association with reduced haemocyte count and reduced protein levels at a time of elevated water temperature. In addition, in a review of disease in marine aquaculture, Sindermann (1968) has stated that mortalities of lobsters infected with *Aerococcus viridans* (var.) *homari* (*Gaffkya homari*) increase sharply if water temperature exceeds 15 °C, and makes the general observation that abnormal temperatures may lower resistance to disease in marine animals. Certainly, temperature is a major determinant of the metabolic and cellular processes and has a pervasive influence on the biology of ectothermic animals, but the extent to which it affects immunocompetence in crustaceans remains largely unknown.

No account was taken of the moult cycle or reproductive cycle in this survey (see Chang & O'Connor, 1983, for molt cycle alterations to metabolism). All animals used were male and all were at intermoult stage, but the precise stage of the cycle, as described by Drach (1939), was not determined. Amongst the many physiological changes that occur at ecdysis is a decrease in the number of circulating haemocytes, which are deployed to ectodermal layers where they contribute to the important process of tanning the new exoskeleton (Vacca & Fingermann, 1983). Since antibacterial activity resides in the haemocytes, a reduction in circulating blood cells may well compromise the host. However, the results presented here are not influenced by the presence of actively moulting crabs. Crabs at moult are soft and easily recognized; also *C. maenas* habitually goes into hiding at ecdysis (usually by burrowing, a strategy that protects it from predators and accidental injury during the 'soft' stage) and in the event that crabs undergo moult in the aquarium, they are rapidly consumed by their relatives.

Every effort was made to select healthy, undamaged animals for each experiment, but the exact immune status of animals collected in the wild is impossible to assess. It has been shown by Smith & Ratcliffe (1980a,b) that, in *C. maenas*, injected particles are phagocytosed or sequestered in nodules in the gills, and the latter event is associated with a decrease in total haemocyte count. Thus it remains possible that some of the low cell counts in individual animals could reflect active involvement of haemocytes in host defence.

In conclusion, data presented here suggest that antibacterial activity is largely affected by extreme water temperatures, rather than showing a graded response over a range of temperatures, and it is likely that this occurs as a result of variation in haemocyte counts and protein levels in the blood. Although haemocyte count (and, therefore, protein concentration) is influenced by temperature, there are other prevailing factors to be taken into account such as reproductive cycle, moult cycle, seasonal availability of food and exposure to potential pathogens which may affect haemocyte numbers. As already noted in the introductory part of this chapter, few seasonal studies have been carried out with respect to crustacean immune responses and this appears to be the first report concerning seasonal variation in antibacterial activity in *C. maenas*.

Chapter 4
Characteristics of the Antibacterial Activity, and its Relationship to the proPO System in *Carcinus maenas*

4.1. Introduction

Antibacterial factors in crustaceans have been the subject of only a few investigations (see Chapter 1, page 46) and, where demonstrated, they are incompletely characterized with regard to titre, thermal stability, divalent cation dependency, and site of origin. Table 1.4, page 48, reveals that a relatively small number of species have been studied and for most there is little information other than that the presence of an antimicrobial effect has been recorded.

The purpose of this chapter is to examine the characteristics mentioned above (i.e., titre, thermal stability, divalent cation requirement, and site of origin) and to explore the hypothesis, stated in Chapter 1, that there is a connection between the antibacterial activity in *Carcinus maenas* haemocytes and the activation of the prophenoloxidase (proPO) activating system. Preliminary work by Söderhäll & Smith (1986b), localising proPO activity and antimicrobial activity to the same cell population (granular haemocytes) makes this a reasonable hypothesis, although to date, no clear relationship has been established between the proPO system and antibacterial activity in arthropods. In crustaceans, conversion of proPO to phenoloxidase can occur via proteolytic cleavage brought about by a naturally occurring serine protease found in the haemolymph (Söderhäll, 1981), and in insects, cleavage of proPO is associated with the release of a peptide of 5,000 Da (Ashida *et al.*, 1974). The present study utilized *in vitro* enzyme assays for phenoloxidase and protease activity as a means of clarifying whether there is any relationship between antibacterial activity and proPO activation in HLS from *C. maenas*.

4.2. Materials and Methods

4.2.1. Preparation of separated cell lysate supernatants

Haemolymph from healthy male crabs was collected into ice-cold marine anticoagulant (MAC) as previously described in Chapter 2 (page 60) and haemocyte populations were separated by density gradient centrifugation using 10 ml of 60 % Percoll (Pharmacia, Uppsala, Sweden) in 3.2 % NaCl, as described in Söderhäll and Smith (1983). Hyaline or granular cell fractions from five animals were pooled into 20 ml citrated cacodylate buffer (CAC), pH 7.0, in polycarbonate tubes before centrifugation at 1,900 g (10 min at 4 °C). Hyaline cell lysate supernatants (HyLS) or granular cell lysate supernatants (GLS) were then prepared, each in 2 ml modified *Carcinus* saline (CS II), as described for HLS in Chapter 2, page 60.

Purity of hyaline cell fractions was checked, using the rapid drop-assay given in Söderhäll and Smith (1983) which utilizes phenoloxidase as a marker for the granular cells (see below, section 4.2.4). Only those fractions which exhibited negative or negligible phenoloxidase activity were used to prepare HyLS samples, since presence of phenoloxidase in any of the hyaline cell bands was interpreted as being indicative of poor cell separation.

4.2.2. Plasma preparation

Two millilitre samples of haemolymph were obtained aseptically in the absence of anticoagulant and were centrifuged immediately at 600 g in sterile polycarbonate tubes (4 °C for 15 min). The plasma supernatants were then transferred to ice-cold, sterile bijoux and used without delay.

4.2.3. Experiments

All the antibacterial assays described in this chapter followed the protocol described in Materials and Methods, Chapter 2 page 63, and used *Psychrobacter immobilis* as the test organism. For titre of activity, HLS was prepared in the usual way (see Chapter 2, page 60), and was standardized to an initial protein concentration of 1.8 mg ml^{-1}. Serial ten-fold dilutions to 10^{-4} in CS II were performed on 1 ml aliquots of HLS, after which 900 μl of each dilution were tested for antibacterial activity. Titre is given as the reciprocal of the highest dilution at which antibacterial activity was detected.

Heat stability was assessed using 10 ml HLS prepared from the pooled haemolymph of 10-12 crabs. Two millilitre aliquots were transferred to sterile bijoux and one sample was retained on ice until needed. Four samples were heat treated at 50 °C, 60 °C or 70 °C for 20 min or at 100 °C for 30 min, cooled, centrifuged at 2,000 g to remove any coagulated proteinaceous material and stored on ice until all samples were ready for use.

Freeze-thaw stability was investigated using 12 ml of HLS prepared from pooled samples taken from 12 animals. Nine hundred microlitre aliquots were transferred to sterile Eppendorfs and two samples were retained for immediate use. The remaining material was stored either at -20 °C or -70 °C, for one week, one month or three months. After these periods, samples were thawed at 20 °C and, prior to testing, were agitated vigorously, using a Whirlimix to dislodge any protein which may have adhered to the walls of the tubes.

Two HLS samples were used in each test for divalent cation dependence, one prepared in 2 ml Ca^{++} depleted CS II and the other in 2 ml CS II which contained 20 mM Ca^{++}. Both preparations were derived from a single pooled sample of haemolymph in MAC, which had been split prior to the first centrifugation.

Assays were carried out using GLS and HyLS, to confirm the results of preliminary investigations by Söderhäll & Smith (1986b) into the location of antibacterial activity in different cell types. Protein levels in GLS and HyLS were approximately an order of magnitude lower than than those in HLS and, to compensate for this, the initial concentration of the bacterial inoculum was reduced by a factor of ten. Plasma from five individual crabs was also tested for antibacterial activity.

All the above experiments were carried out at 20 °C with an incubation time of 4 h and were repeated three times. Protein levels in all HLS, GLS, HyLS and plasma samples (including heat-treated material) were determined as described in Chapter 2, page 64. Concentrations of protein in fresh HLS ranged from 1.5 - 1.8 mg ml^{-1} unless otherwise indicated. Plasma protein in individual crabs was variable and ranged from 32.0 - 75.0 mg ml^{-1}.

4.2.4. Enzyme assays

Concurrent *in vitro* assays for phenoloxidase activity (see Chapter 2, page 63) and protease activity were performed on each sample of HLS used in the heat and freeze-thaw stability test. It is important to note that calculations of phenoloxidase and protease activities for heat-treated samples are based on protein concentrations measured subsequent to heat treatment, cooling and centrifugation. Average protein levels in HLS after heat treatment were 1.43 ± 0.05, 1.23 ± 0.05, 0.93 ± 0.06 and 0.53 ± 0.15 mg^{-1} for the 50 °C, 60 °C, 70 °C and 100 °C treatments respectively.

Protease activity was measured spectrophotometrically at 405 nm using the synthetic chromogenic peptide S2337 (Kabi Vitrum) as substrate (Söderhäll, 1983). One hundred microlitres of HLS were pre-incubated with 100 μl of elicitor (lipopolysaccharide (LPS) from *E. coli* 0111: B4, phenolic extraction; Sigma. Poole, Dorset) (1 mg ml^{-1} in Tris-HCl buffer, pH 8.0) for 10 min at 20 °C prior to addition

of 100 μl of the chromogenic peptide S2337 (also 1 mg ml^{-1} in Tris-HCl buffer, pH 8.0) together with 200 μl Tris-HCl buffer (pH 8.0). After incubation for 1 h at 20 °C the reaction was terminated by the addition of 100 μl 50 % acetic acid. Controls were set up using Tris-HCl buffer as a substitute for LPS. Protease activity was expressed as nanomoles p-nitroaniline (nmol PNA) released h^{-1} mg^{-1} protein and was calculated according to the formula given below (modified from the formula supplied by Kabi Vitrum with the chromogenic peptide S2337).

$$\text{Protease activity} = \frac{A \times V \times 10^9}{v \times \varepsilon \times t \times c}$$

A = absorbance at 405 nm

V = total volume of reaction mixture in litres

v = total volume of HLS tested in litres

ε = molar absorptivity of p-nitroaniline at 405 nm, given as 9.600×10^3

t = reaction time in hours

c = concentration of protein in HLS in mg per litre.

10^9 to convert moles to nanomoles

A rapid drop assay for phenoloxidase (Smith & Söderhäll, 1983) was used to check the purity of the different cell fractions. Fifty microlitres of the test lysate supernatant, 50 μl trypsin (Sigma) (from porcine pancreas; 1 % in CS II) and 50 μl L-dopa (3 mg ml^{-1} in CS II) were incubated together in clean petri dishes at room temperature. Any samples that had turned pink after the elapse of 30 min were judged to contain phenoloxidase (i.e were contaminated with granular cell material). Appropriate controls were set up using CS II as a substitute for the elicitor.

4.3. Results

4.3.1. Requirement for divalent cations

Antibacterial activity was found to be independent of Ca^{++} ions (Table 4.1). The bacterial response appeared almost identical for both Ca^{++} depleted and Ca^{++} supplemented HLS preparations in two of the three repeat experiments: survival index values (SI) close to zero were attained within one hour (Table 4.1). For the third experiment, antibacterial activity was slightly stronger with Ca^{++} depleted buffer than with the Ca^{++} supplemented buffer (Table 4.1), but activity was nevertheless extremely strong in the latter (SI 3.3 after 1 h and SI 1.2 after 4 h) when compared with the control. Furthermore, the antibacterial effect was judged to be independent of Mg^{++} since HLS was routinely prepared in a Mg^{++} free buffer. It was noted that in the Ca^{++} depleted controls the bacteria survived less well than in the Ca^{++} supplemented controls. However, at neither of the time points did SI values for these controls drop below 95 and the difference in SI between the controls and experimentals remained consistantly large in all cases.

4.3.2. Thermal stability

Heat treatment

Table 4.2 shows a progressive increase in SI with increasing temperature. After 4 h incubation, SI values of < 0.01, 4.7 ± 1.1, 4.1 ± 1.4 and 37.1 ± 2.5 were recorded for samples heat treated at 50 °C, 60 °C, 70 °C and 100 °C respectively. These values represent a gradual loss of antibacterial activity; nevertheless, an SI of 37 after treatment to 100 °C indicates retention of more than 60 % of the activity recorded for fresh HLS. With respect to the possible relationship between antibacterial activity and phenoloxidase activity, it was observed that whilst increasing treatment temperature up to 60 °C resulted in a slight decline in antibacterial effect (SI 4.7 ± 1.1 compared

Table 4.1. Effect of calcium on the antibacterial activity of HLS[1] against *Psychrobacter immobilis*

	SI[2]	
Treatment[3]	1 hour	4 hours
HLS + Ca^{++}	< 0.01	< 0.01
	3.3	1.2
	< 0.01	< 0.01
Control + Ca^{++}	127.2	142.0
	152.5	170.4
	114.0	166.6
HLS - Ca^{++}	< 0.01	< 0.01
	< 0.01	< 0.01
	< 0.01	< 0.01
Control - Ca^{++}	155.3	102.1
	97.5	99.1
	116.6	110.8

[1]HLS: Haemocyte lysate supernatant prepared as described in Materials and Methods, Chapter 2, page 60.

[2]SI: Survival index calculated as described in Materials and Methods, Chapter 2, page 63.

[3]Treatment: Ca^{++} depleted and Ca^{++} supplemented (20 mM) buffers were used for the preparation of HLS, as described in Materials and Methods page 101.

Table 4.2. Antibacterial activity and phenoloxidase activity in heat-treated HLS[1]

	SI[2]		Phenoloxidase activity[3] (units min^{-1} mg $protein^{-1}$)	
Treatment	1 hour	4 hours	LPS treated[4]	Buffer control[5]
Fresh untreated	1.9 ± 0.9	< 0.01	39.9 ± 10.2	20.0 ± 9.8
50 °C (20 min)	5.3 ± 3.1	<0.01	100.0 ± 40.0	41.2 ± 19.8
60 °C (20 min)	22.0 ± 6.0	4.7 ± 1.1	138.0 ± 10.0	61.0 ± 28.7
70 °C (20 min)	51.7 ± 11.8	4.1 ± 1.4	69.5 ± 9.2	50.0 ± 10.0
100 °C (30 min)	57.0 ± 10.9	37.1 ± 2.5	< 10.0	< 10.0
Control[6]	130.9 ± 23.7	201.9 ± 42.7		

[1]HLS: Haemocyte lysate supernatant prepared as described in Materials and Methods, Chapter 2, page 60.

[2]SI: Survival index for *Psychrobacter immobilis*, calculated as given in Materials and Methods, Chapter 2, page 63.

[3]Phenoloxidase activities were calculated from absorbances read at 490 nm and expressed as units min^{-1} mg $protein^{-1}$. One unit is the amount of enzyme activity which gives an increase in absorbance of 0.001 at 490 nm at 20 °C under the experimental conditions.

[4]HLS was pre-incubated with LPS (1 mg ml^{-1}) for 20 min before addition of L-dopa (3 mg ml^{-1}), as described in Materials and Methods, Chapter 2, page 63.

[5]Controls for phenoloxidase assays contained modified *Carcinus* saline (CS II) as a substitute for LPS.

[6]Controls for antibacterial activity assays contained equal parts of CS II and marine broth as a substitute for HLS.

Values are means ± standard deviations (n = 3).

with SI < 0.01 for fresh HLS), corresponding phenoloxidase assays showed an enhancement of enzyme activity (Table 4.2). However, for HLS heat-treated to 70 °C and 100 °C, phenoloxidase activity was reduced by *circa* 50 % and 90 % respectively compared with that recorded for 60 °C (Table 4.2), although antibacterial activity was still measurable at the higher temperatures.

Protease activity increased after treatment at 50 °C and 60 °C (from 19.95 ± 1.3 nmol PNA released h^{-1} mg $protein^{-1}$ for fresh HLS, to 26.96 ± 0.25 and 24.55 ± 1.3 nmol PNA for 50 °C and 60 °C respectively) whilst antibacterial activity showed a gradual decline (Table 4.3). A reduction in protease activity to 15.74 ± 5.74 nmol PNA released h^{-1} mg $protein^{-1}$ occurred after treatment at 70 °C although antibacterial activity showed little change from the value recorded at 60 °C (Table 4.3). Further reduction in protease level occurred after treatment at 100 °C and was accompanied by an increase in SI for *P. immobilis* (Table 4.3).

Freeze treatment

With respect to freeze-stability, Table 4.4 shows that after 1 h the antibacterial effect of HLS was markedly enhanced following storage at -70 °C for three months; the SI for *P. immobilis* challenged with freeze-thawed HLS was 4.8 ± 2.7 whereas an SI of 38.6 ± 2.0 was recorded after challenge with fresh material. After 4 h the same samples gave SI values of almost zero and 4.2 ± 1.9 respectively. Enhanced antibacterial activity was also seen at 1 h incubation with intermediate periods of storage at -70 °C, but the effect was less pronounced (see Table 4.4). Similar results were obtained with material frozen at -20 °C (Table 4.5). Again, associated phenoloxidase assays failed to reveal any relationship between enzyme activity and antibacterial effect (Tables 4.4 & 4.5). Approximately 30 % and 60 % of enzyme activity was lost after three months storage at -20 °C and -70 °C respectively, although there was no parallel increase in SI of *P. immobilis* (Tables 4.4 & 4.5).

Table 4.3. Protease activities in heat-treated HLS[1].

	SI[2]		Protease activity[3] (nmol PNA released h^{-1} mg $protein^{-1}$)	
Treatment	1 hour	4 hours	LPS treated[4]	Buffer control[5]
Fresh untreated	1.9 ± 0.9	< 0.01	19.9 ± 1.3	2.2 ± 0.3
50 °C (20 min)	5.3 ± 3.1	<0.01	26.9 ± 0.3	5.7 ± 0.1
60 °C (20 min)	22.0 ± 6.0	4.7 ± 1.1	24.5 ± 1.3	7.7 ± 0.4
70 °C (20 min)	51.7 ± 11.8	4.1 ± 1.4	15.7 ± 5.4	6.1 ± 2.3
100 °C (30 min)	57.0 ± 10.9	37.1 ± 2.5	7.5 ± 3.2	1.7 ± 0.9
Control[6]	130.9 ± 23.7	201.9 ± 42.7		

[1]HLS: Haemocyte lysate supernatant prepared as described in Materials and Methods, Chapter 2, page 60.

[2]SI: Survival index for *Psychrobacter immobilis* calculated as given in Materials and Methods, Chapter 2 page 63.

[3]Protease activities were calculated from absorbances read at 405 nm and expressed as nanomoles of p-nitroaniline (PNA) released h^{-1} mg $protein^{-1}$ according to the equation given in Materials and Methods, page 103.

[4]HLS was pre-incubated with LPS (1mg ml^{-1}) for 20 min before addition of the chromogenic peptide S2337 (1mg ml^{-1}) as described in Materials and Methods, page 102.

[5]Controls for the protease assay contained Tris-HCl buffer (pH 8.0) as a substitute for LPS.

[6]Controls for antibacterial activity assays contained equal parts of modified *Carcinus* saline (CS II) and marine broth as a substitute for HLS.

Values are means ± standard deviations (n = 3).

Table 4.4. Antibacterial activity and phenoloxidase activity in HLS stored at -70°C

	SI[2]		Phenoloxidase activity[3] (units min^{-1} mg $protein^{-1}$)	
Storage time	1 hour	4 hours	LPS treated[4]	Buffer control[5]
Nil	38.6 ± 2.0	4.2 ± 1.9	160.2 ± 60.4	10.8 ± 5.1
1 week	19.2 ± 9.3	4.8 ± 4.2	127.4 ± 15.0	10.5 ± 2.3
1 month	27.4 ± 2.3	4.8 ± 2.4	119.4 ± 30 .2	13.2 ± 3.0
3 months	4.8 ± 2.7	< 0.01	31. 6 ± 3.0	23.7 ± 4.1

[1]HLS: Haemocyte lysate supernatant prepared as described in Materials and Methods, Chapter 2, page 60.

[2]SI: Survival index for *Psychrobacter immobilis* calculated as given in Chapter 2, page 63. For the sake of clarity, values for controls are not shown, but all controls (containing modified *Carcinus* saline (CS II) as a substitute for HLS) showed growth, with SI > 100.

[3]Phenoloxidase activities were calculated from absorbances read at 490 nm and expressed as units min^{-1} mg $protein^{-1}$. One unit is the amount of enzyme activity which gives an increase in absorbance of 0.001 at 490 nm at 20 °C under the experimental conditions.

[4]HLS was pre-incubated with LPS (1mg ml^{-1}) for 20 min before addition of L-dopa (3mg ml^{-1}).

[4]Controls for the phenoloxidase assay contained CS II as a substitute for LPS.

Values are means ± standard deviations (n = 3).

Table 4.5. Antibacterial activity and phenoloxidase activity in HLS[1] stored at -20°C

	SI[2]		Phenoloxidase activity[3] (units min^{-1} mg $protein^{-1}$)	
Treatment	1 hour	4 hours	LPS treated[4]	Buffer control[5]
Fresh	38.6 ± 2.0	4.2 ± 1.9	106.2 ± 60.4	10.8 ± 5.1
1 week	31.3 ± 3.7	4.8 ± 1.5	149.1 ± 15.3	10.6 ± 2.5
1 month	29.6 ± 2.4	3.5 ± 1.2	208.4 ± 14.6	26.8 ± 6.3
3 months	16.1 ± 2.1	< 0.01	66.2 ± 13.0	41.4 ± 4.4

[1]HLS: Haemocyte lysate supernatant prepared as described in Materials and Methods, Chapter 2, page 60.

[2]SI: Survival index for *Psychrobacter immobilis* calculated as given in Chapter 2, page 63. For the sake of clarity, values for controls are not shown but all controls (containing modified *Carcinus* saline (CS II) as a substitute for HLS) showed growth, with SI > 100.

[3]Phenoloxidase activities were calculated from absorbances read at 490 nm and expressed as units min^{-1} mg $protein^{-1}$. One unit is the amount of enzyme activity which gives an increase in absorbance of 0.001 at 490 nm at 20 °C under the experimental conditions.

[4]HLS was pre-incubated with LPS (1mg ml^{-1}) for 20 min before addition of L-dopa (3mg ml^{-1}).

[4]Controls for the phenoloxidase assay contained CS II as a substitute for LPS.

Values are means ± standard deviations (n=3)

Protease activities in fresh samples used in the freeze-treated experiments were very high compared with the activities recorded for fresh samples used in heat treated experiments (Table 4.3 &.4.6). After freezing for one week and one month, activities in the material stored at -20 °C were amplified but at three months had dropped to low levels (*circa* 11.2 nmol PNA released h^{-1} mg $protein^{-1}$ compared with 49.2 nmol PNA released for the fresh sample) (Table 4.6). Antibacterial activity in the same samples, on the other hand, was enhanced (see above and Table 4.3). Haemocyte lysate supernatants stored at -70 °C showed reduced protease activity after one week, enhanced activity after one month and again low levels after three months (Table 4.6) whilst the antibacterial effect in the same samples was high (see above and Table 4.3).

4.3.3. Titre

The antibacterial factor present in *C. maenas* haemocytes was found to be active at high titre, 10^4 , which represents a protein concentration of *circa* 2 μg ml^{-1} (Figure 4.1). Although the trend was for SI to increase (i.e., antibacterial activity to weaken) with increasing titre, it was still possible to detect a reduction of SI, compared with the control, at a titre of 10^4 after 4 h incubation. Initially controls for the titre of activity experiments were set up in the usual way (see Chapter 2, page 63) with equal parts of marine broth and CS II as the culture medium. However, this did not allow for the possibility that highly diluted HLS samples may provide inadequte nutrition for the bacteria; to allow for this the experimental protocol was modified to include an additional control comprising CS II only. The bacteria survived for the duration of the experiment, giving SI values of 103 ± 4 (Figure 4.1) over 4 h, but did not achieve the growth observed in the broth-buffer mixtures.

4.3.4. Location of antibacterial activity

Comparison of lysate supernatants from granular cells, hyaline cells and mixed cells (GLS, HyLS and HLS respectively) revealed that antibacterial activity resides

Table 4.6. Protease activities in HLS[1] stored at -20 °C and -70 C

	Protease activity[2] (nmol PNA released h^{-1} mg $protein^{-1}$)					
	HLS stored at -20 °C		HLS stored at -70 °C		Fresh HLS	
Storage time	LPS treated[3]	Buffer control[4]	LPS treated[3]	Buffer control[4]	LPS treated[3]	Buffer control[4]
Zero					49.2 ± 1.9	5.2 ± 1.0.
1 week	55.6 ± 2.2	8.8 ± 1.0	37.7 ± 0.4	5.8 ± 0.5		
1 month	153.6 ± 20.8	14.2 ± 1.7	61.6 ± 5.8	5.2 ± 0.6		
3 months	11.2 ± 2.0	10.0 ± 1.6	9.8 ± 1.6	8.4 ± 3.2		

[1]HLS: Haemocyte lysate supernatant prepared as described in Materials and Methods, Chapter 2, page 60.

[2]Protease activities were calculated from absorbances read at 405 nm and expressed as nanomoles of p-nitroaniline (PNA) released h^{-1} mg $protein^{-1}$ according to the equation given in Materials and Methods, page 103.

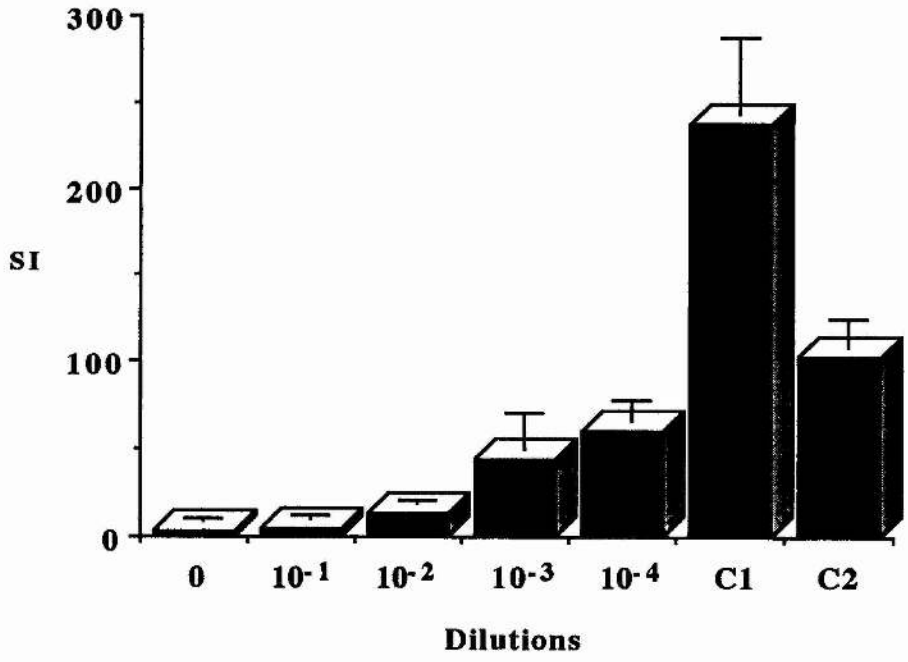
[3]HLS was pre-incubated with lipopolysaccharide (LPS;1mg ml^{-1}) for 20 min before addition of the chromogenic peptide S2337 as described in Materials and Methods, page 102.

[4]Controls for the protease assay contained Tris-HCl (pH 8.0) as a substitute for LPS.

Values are means ± standard deviations (n = 3).

Figure 4.1

Figure 4.1. Titre of activity of haemocyte lysate supernatant (HLS) against *Psychrobacter immobilis* after four hours. The bacteria were incubated in HLS (prepared as described in Materials and Methods, Chapter 2, page 60) that had been subjected to serial ten fold dilutions in modified *Carcinus* saline (CS II) up to a dilution of 10^{-4}. For each dilution, survival indices (SI) were calculated for *P. immobilis*, as described in Materials and Methods, Chapter 2, page 63. C1 and C2 are the two sets of controls. C1 contained equal parts of CS II and marine broth. C2 contained CS II only. Values are means ± standard deviations (n=3).


300
200
100
0
SI
0
10-1
10-2
10-3
10-4
C1
C2
Dilutions

exclusively in GLS, with an effect almost identical to that recorded for HLS (Table 4.7). By contrast, the bacteria thrived in HyLS (SI *circa* 300 after 4 h), as can be seen in Table 4.7 which gives data from one representative experiment. Means and standard deviations were not calculated for all three experiments due to large differences in the bacterial growth observed in HyLS (SI values ranging from 159 to 399), although survival indices in GLS remained consistently low.

An SI of 52.6 ± 13.3 (n = 5) was recorded after incubation of *P. immobilis* in plasma prepared by centrifugation of whole haemolymph at 600 g for 15 min (Table 4.8). These plasma samples also showed low levels of phenoloxidase activity (Table 4.8). To clarify whether the method of plasma preparation may have influenced SI values, additional experiments were carried out using whole haemolymph centrifuged at 500 g or 1,900 g for 15 min. As Table 4.8 shows, increasing the centrifugal force appeared to increase the antibacterial activity in the plasma sample. The 500 g plasma gave an SI of 201 ± 28.0 whereas the 1,900 g plasma gave an SI of 0.5 ± 0.1 (Table 4.8). Phenoloxidase activity was also affected; an approximate ten fold increase in activity was observed in the 1,900 g plasma when compared with the 500 g plasma (Table 4.8).

4.4. Discussion

Antimicrobial factors represent one component of the immune response and have been described for a number of invertebrate groups (see review by Ratcliffe *et al.*, 1985). Within the Crustacea, observations are restricted to about seven species, one of which, *Callinectes sapidus*, shows antiviral activity (McCumber & Clem, 1977) and another, *Pacifastacus leniusculus* (Nyhlén & Unestam, 1980), antifungal activity. For the rest (*Panulirus argus, Panulirus interruptus, Homarus americanus, Penaeus monodon* and *Carcinus maenas*), none of the recorded factors have been

Table 4.7. Effect of cell lysate supernatants on the survival of *Psychrobacter immobilis*

Cell preparation	SI[1] at 4 hours
HLS[2]	3.8
HyLS[3]	304.8
GLS[4]	5.3
Control[5]	267.8

[1]SI: Survival index, calculated as given in Materials and Methods, Chapter 2, page 63.

[2]HLS: Haemocyte lysate supernatant from unseparated cells, prepared as described in Materials and Methods, Chapter 2, page 60.

[3]HyLS: Hyaline cell lysate supernatant, prepared as described in Materials and Methods, page,100.

[4]GLS: Granular cell lysate supernatant, prepared as described in Materials and Methods, page 100.

Values are results from a typical experiment. The same trend was observed in all replicated experiments (n = 3).

Table 4.8. Effect of plasma on the survival of *Psychrobacter immobilis*

Preparation[1]	SI at 4 hours[2]	Phenoloxidase activity[3] (units min^{-1} mg $protein^{-1}$)
500 g	201.0 ± 28.0	8.0 ± 1.0
600 g	52.6 ± 13.2	14.0 ± 2.0
1900 g	0.5 ± 0.1	90.0 ± 21.1
Control[4]	493.0 ± 85.0	

[1]Haemolymph from individual crabs was centrifuged for 15 minutes under different conditions of centrifugal force, as described on page 115.

[2]SI: Survival index calculated as given in Materials and Methods, Chapter 2, page 63.

[3]Phenoloxidase activities were calculated from absorbances read at 490 nm and expressed as units min^{-1} mg $protein^{-1}$. One unit is the amount of enzyme activity which gives an increase in absorbance of 0.001 at 490 nm at 20 °C under the experimental conditions.

[4]Controls contained marine broth as a substitute for plasma.

Values are means ± standard deviations (n=5)

fully characterized and none have been purified (see review by Smith & Chisholm, 1992).

The present study, using *C. maenas,* demonstrates that an antibacterial factor (or factors) exists in the haemocytes and is absent from plasma. The factor(s) are also independent of calcium ions, heat stable and active at high titre.

Little is known about the biochemical and biophysical properties of antibacterial factors in crustaceans. A factor in one species, *P. argus*, which has been more fully investigated, shows no requirement for divalent cations (Evans *et al.*, 1968) and an equivalent finding is reported in the present study for *C. maenas*. The small differences noted, in experimental tubes, between the two treatments in one set of experiments (Table 4.1) may have been due to experimental error in plating out or could have been a consequence of unequal division of the suspended cell sample prior to preparing the two different lysates. Nevertheless, the antibacterial effect in this particular instance was still very powerful (Table 4.1). Bacterial growth *in vitro* was compromised in Ca^{++} depleted buffers (see Table 4.1 for control values) and this may have contributed in part to the low SI values recorded for Ca^{++} depleted HLS. A reasonable interpretation of these results would be that, using this experimental method, Ca^{++} does not appear to have a marked influence on the antibacterial activity in *C. maenas* HLS.

The experimental protocol used here (i.e. preparing HLS in Ca^{++} supplemented or Ca^{++} depleted buffer) is not perhaps ideal and overlooks the possibility that, in spite of collecting haemolymph in an anticoagulant which contains a chelating agent, EDTA, some cell-derived divalent cations may be present in the final HLS, regardless of the buffer constituents. An alternative approach might be to treat HLS with EDTA and to then dialyse the resultant mixture against Ca^{++} free buffer. An attempt at dialysis was made, but it was impossible to execute this and maintain sterility in the

final sample. According to Evans *et al.* (1968), the bactericidin in *P. argus* was not appreciably diminished by treatment with EDTA, but how their samples were prepared is not altogether clear.

The freeze-stability of the antibacterial factor reported here also occurs in *P. argus* (Evans *et al.*, 1968). However, the reason for enhancement of antibacterial activity in *C. maenas* HLS, after prolonged freezing, remains enigmatic. For crustaceans as a group, there has been no attempt to define the mechanism(s) by which antibacterial factors are regulated, so whether these observations result from a loss of modulators or are due to some separate effect, such as conformational change of bioactive factors, is unknown.

With respect to heat stability, the antibacterial factor in *C. maenas* haemocytes appears stable to 70 °C and has measurable residual activity after heating to 100 °C. The factor or factors in *C. maenas* are thus more robust than those present in *P. argus* (Evans *et al.*, 1968) and *P. interruptus* (Evans *et al.*,1969a) in which the antibacterial effect is lost at 65°C.

Antibacterial activity in *C. maenas* can also be measured at high titre, suggesting that the factor responsible is potent. Over the four hour incubation period the SI of 103 ± 4 for the buffer-only controls, whilst not demonstrating the same growth as the nutrient-supplemented controls, showed that the bacteria survived for that time interval. Nevertheless, it remains possible that in a nutritionally depleted environment the bacteria may have been more susceptible to small amounts of the active factor and so perhaps the results relating to the highest titre (10^4) should be viewed critically.

The mode of action of the antibacterial factor(s) is unclear, but from the evidence presented here it seems unlikely that antibacterial activity in *C. maenas* haemocytes could be attributed to agglutination. The features of heat stability, activity at high titre and no requirement for divalent cations distinguish antibacterial activity *per se,* from

agglutination in other crustaceans. In general, the latter response is inactivated at 60 °C, operates at low titre, and is dependent on divalent cations (see review by Smith & Chisholm, 1992 and Table 1.2). However, the possibility that bacterial agglutination may contribute to the antibacterial effect should not be dismissed and is a consideration that will be addressed in Chapter 5.

The fact that antibacterial activity resides in the granular cells, which also contain the prophenoloxidase activating (proPO) system, gave rise to the initial question of whether the appearance of this activity is dependent on activation of the proPO system. From the results of the experiments described here for *C. maenas,* the relationship of the proPO system to antibacterial activity in the haemocytes remains equivocal. It is clear that phenoloxidase itself is not responsible for the antibacterial effect since reduction of bacterial number in test solutions persists even when phenoloxidase activity has been lost by heating, or has been substantially reduced by freezing.

As for the relationship between protease activity and antibacterial activity, the results from heat treated samples alone would encourage further investigation of a link with this enzyme (high protease activities were broadly associated with low survival indices and vice versa), but taken in conjunction with the results from frozen HLS, it is reasonable to conclude that the antimicrobial effect is not due to protease activity itself. However, the proPO system is a complex cascade of enzymes which generates several bioactive molecules, including peptides (Söderhäll & Smith, 1986a) which could contribute to the antibacterial effect in this animal. Peptides are known to have antibacterial properties in insects and other animals (Boman, 1991), so an antibacterial role for other factors in the proPO system of *C. maenas* should not be excluded at this stage.

The reason for large differences in baseline protease levels between fresh HLS samples prepared for heat treatment and those prepared for freezing is unknown. Unlike many experimental animals (e.g. insects) which can be reared in controlled laboratory conditions, specimens of *C. maenas* were collected from the wild and it is likely that a number of differences exist between batches of animals, even after a period of acclimation in the aquarium. It is also possible that some activity was lost from fresh HLS used in the heat treatment experiments since samples were maintained on ice and not processed until all heat treated aliquots were ready for testing (*circa* 40 minutes).

The absence of antibacterial activity in plasma, reported here, supports a previous finding by White *et al.*, (1985), although the data presented in Table 4.8 shows that observations are heavily dependent on the method of plasma preparation. Crustacean granular cells exocytose readily in the presence of endotoxins *in vitro* (Smith & Söderhäll, 1983) and, from the results of the present study, it seems that the extent to which these cells cells lyse or exocytose is affected by the centrifugal force they experience during plasma preparation. In this case, contamination of plasma preparations by granular cell products, as marked by phenoloxidase levels, clearly influenced the results of antibacterial assays, giving low SI values for the test organisms.

Overall, this set of experiments presents some physicochemical characteristics of the antibacterial factor in HLS and demonstrates that there is no direct link between antibacterial activity and two key enzymes (a serine protease and phenoloxidase) in the proPO system.

Chapter 5
Agglutination and Lysozyme Studies for *Carcinus maenas*

5.1 Introduction

It has been shown in the preceding chapters that for some bacteria (e.g. *Psychrobacter immobilis* and *Pseudomonas* 1-1-1) the antimicrobial activity that resides in *Carcinus maenas* haemocytes is profound. However, the mechanism by which this occurs has not yet been determined and whether the observed phenomena are due to lysis, bacteriostasis or agglutination is not known.

Antibacterial activity has been recorded in several invertebrate groups, from sponges to tunicates, although in general there has been little distinction as to the bacteriocidal or bacteriostatic nature of the factors involved (see reviews by Ratcliffe *et al.*, 1985; Smith & Chisholm, 1992; Söderhäll & Cerenius,1992). One notable exception lies in the excellent work of Boman and co-workers who have revealed the mode of action, and also carried out amino acid sequencing, for a battery of antibacterial factors found in insects (Boman, 1986, 1991, for review). These antibacterial molecules include the the attacins, which alter cell membrane permeability in bacteria, and the cecropins, which are lytic for both Gram-positive and Gram-negative organisms (Boman, 1986).

Another factor, lysozyme, first reported to occur in tears by Alexander Fleming (1922) and subsequently reported in several invertebrates (see reviews by Ratcliffe *et al.*, 1985), is bacteriolytic and specifically attacks the ß, 1-4 glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine in the cell walls of Gram-positive bacteria (Salton, 1957). Lysozyme has been the subject of much investigation, particularly in molluscs, where lysozyme or lysozyme-like activity has been identified in many species (Mc Henery & Birkbeck, 1982; McHenery *et al.*, 1979, 1986) and in several different tissues, including haemolymph (Cheng & Roderick, 1974; Ottaviani, 1991). As for the crustaceans, apart from the work of Fenouil & Roch (1992), who

have found lysozyme in six species of freshwater crayfish, there is no evidence, thus far, to suggest that lysozyme contributes substantially to host defence in crustaceans.

Antimicrobial factors are also present in the haemolymph of marine annelids and one animal, *Glycera dibranchiata,* has been extensively researched in this respect (Chain & Anderson, 1983a,b,c,d). It has been shown that the factor in *G. dibranchiata,* which is non-lytic and preferentially attacks Gram-negative bacteria, appears to operate by a two-step process - an initial reversible binding to the bacterial cell surface followed by a "killing reaction" (Chain & Anderson, 1983b). Antibacterial activity has been reported in other marine annelids such as *Phascosoloma gouldii* and *Dendrostomum zostericolum* (see for example, Bang & Krassner, 1958; Evans *et al.,* 1969c; Johnson & Chapman, 1970a) but the mechanism involved has not been elucidated. Likewise, in the studies of echinoderms (Wardlaw & Unkles, 1978; Service & Wardlaw, 1984, 1985), the antibacteral activity of coelomic fluid (due to the pigment echinochrome A) is generally referred to as bactericidal, although whether this is due to lysis or arises via some other mechanism is not certain.

As previously observed in Chapter 1, bacterial agglutination has been recorded in only a few crustaceans. A clear role for bacterial agglutination as part of the host defence system is not difficult to envisage, yet it has attracted little research interest. The work in this chapter is intended to clarify firstly whether antibacterial activity in *C. maenas* haemocye lysate supernatants can be attributed to agglutination, although from the results presented in Chapter 4, it seems unlikely that this would be the case (see Chapter 4, page 119) and secondly to determine whether the antibacterial factor is due to lysozyme or lysozyme-like activity.

5.2. Materials and Methods

5.2.1.Bacterial agglutination assays

The bacteria used in these assays were *Psychrobacter immobilis, Photobacterium phosphoreum, Pseudomonas 3-1-1, Vibrio anguillarum,* (serotypes I & II), BS 68, BS 66, *Planococcus citreus* and virulent and avirulent strains of *Aerococcus viridans* (var.) *homari* (*Gaffkya homari*) (see Chapter 2, Tables 2.1 & 2.2, pages 57 & 58 for colony characteristics). Bacteria were cultured in marine broth, according to their requirements, washed twice in sterile 3.2 % NaCl (pH 7.0) and resuspended in 3.2 % NaCl. After the first wash, all bacterial suspensions were adjusted to an absorbance 0.5 at 570 nm and after the second wash were resuspended in one half the original volume of 3.2 % NaCl to give concentrations of *circa* 10^9 bacteria ml^{-1}.

Agglutination assays were carried out in round bottomed microtitre plates. HLS was prepared in modified *Carcinus* saline (CS II), following the method described in Chapter 2, page 60, and samples were standardized to a protein concentration of 1.2 mg ml^{-1}. Serial two-fold dilutions in CS II were performed on 100 μl aliquots of HLS in microtitre plates and 100 μl of bacterial suspension was added to each well. Controls comprised 100μl bacterial suspension and 100μl CS II. Plates were incubated overnight (12-15 h) at 20 °C or 4 °C.

Plates were examined for agglutination reactions using the naked eye, x 30 magnification and also with an agglutination package on a Dynatech plate reader at 570 nm. Negative agglutination was scored when clearly identifiable pellets had settled in the base of the wells. Positive agglutination was scored when bacterial clumps remained in a diffuse suspension. Titre is given as the reciprocal of the highest dilution at which agglutination was detected. Assays were repeated at least five times for each bacterial species.

5.2.2. Removal of agglutination by adsorption with LPS

Nine hundred microlitres of HLS were incubated with 10μl lipopolysaccharide (LPS: 100 mg ml^{-1} and 10 mg ml^{-1}) for 30 min and then ultracentrifuged at 100,000 g for 30 min at 4 °C to remove complexed material. The supernatant was used for agglutination assays as described above, using *P. immobilis* as the test organism and an incubation temperature of 20 °C. Each experiment compared LPS-treated and untreated HLS from the same initial sample, using 100 μl bacteria and 100μl CS II as a control. The experiment was repeated three times with different HLS samples.

5.2.3. Removal of antibacterial activity by adsorption with LPS and bacteria

Haemocyte lysate supernatants were incubated for 30 min with LPS at a final concentration of 1 mg ml^{-1} and with *P. immobilis* at a final concentration of 5 x 10^9. Lipopolysaccharide-treated samples were then ultracentrifuged as described in section 5.2.2. Bacteria-treated samples were centrifuged at 2,000 g for 15 min and then filtered through a Swinnex Millipore filter (0.2 μ pore size). Nine hundred microlitre aliquots of treated and untreated HLS were used for antibacterial assays against *P. immobilis* at 20 °C, using an incubation period of 4 h as described in Materials and Methods, Chapter 2, page 63. Controls comprising equal parts of marine broth and CS II were set up and the experiment was repeated with three different HLS samples. Lipopolysaccharide solutions were heated to boiling point for 30 min and then cooled, prior to addition to HLS. Contamination controls for treated HLS were plated out during each experiment.

5.2.4. Lytic activity in HLS

Haemocyte lysate supernatants, prepared in the usual way, were tested for lytic activity and growth inhibition using bacterial lawn assays and turbidometric studies.

For growth inhibition, marine agar plates were prepared with a soft agar overlay, to which had been added 1 ml of a 24 h culture of *P. immobilis*. Wells were prepared in the agar using a number 1 cork borer and 10 μl HLS or CS II (as a control) were added to each well. Plates were incubated at 20 °C for 24 h and examined for evidence of bacterial growth inhibition. When present, this manifests itself as a clear zone in the agar around the experimental well.

To determine whether lysis was present, marine agar plates were prepared in the same way, except that the bacterial lawn was allowed to develop before HLS was added to the wells. Plates were again incubated at 20 °C for 24 h before examining for the presence of lysis.

For turbidometric measurements, 500 μl HLS and 500 μl of standard *P. immobilis* suspension were incubated together in experimental tubes, with equal parts of marine broth and CS II subtituted for HLS in controls. Turbidity changes were measured initially at 10, 20, 30 and 120 s, after which the tubes were incubated for 4 h, with further turbidity readings at the end of this period. At the end of the experiment, the numbers of viable bacteria in test and control solutions were determined by serial dilutions and plating out on marine agar. Alterations in turbidity were recorded as a percentage of initial turbidity readings at 10 s and the survival index (SI) was calculated for the spread plates as described in Methods and Materials, Chapter 2, page 63.

5.2.5. Lysozyme activity in HLS

Haemocyte lysate supernatants and hyaline cell lysate supernatants (HyLS) were prepared in the usual way (see Materials and Methods, Chapter 2, page 60 and Chapter 4, page 10), but cell pellets were finally homogenized in 0.06 M phosphate buffer supplemented with 0.1 M NaCl (pH 6.4), instead of CS II.

The salt supplemented phosphate buffer was tested for suitability for lysozyme assays. Hen egg-white lysozyme (HEWL) (Difco) was prepared in phosphate buffer at concentrations of 500, 100, 50, 25, 2.5, and 1.25 μg ml^{-1}, and a standard suspension of *Micrococcus luteus* cell walls (Sigma. Poole, Dorset) was prepared to an absorbance of 0.15 at 570 nm. Experimental tubes contained 900 μl *M. luteus* suspension and 100 μl HEWL solution (giving final concentrations of HEWL of 50, 10, 5, 2.5, 0.25, and 0.125 μg ml^{-1}). Controls contained 900 μl *M. luteus* and 100 μl phosphate buffer, and phosphate buffer was used as a blank. Experimental and control tubes were incubated at 30 °C, with shaking, for 15 min and absorbances were read at 600 nm. Lysis in experimental tubes was expressed as a percentage of the controls:

$$\frac{\text{Control}_{A600} - \text{Experimental}_{A600}}{\text{Control}_{A600}} \times 100$$

The results were used to construct a standard curve for HEWL activity, so that any lytic activity found in HLS or HyLS could be expressed as HEW equivalents. Log concentration of HEWL was plotted against absorbance of *M. luteus* suspensions after incubation for 15 min.

The above protocol was used to test for lysozyme-like activity in HLS and HyLS by substituting lysate supernatants for HEWL. Changes in absorbance were read after 15 min and then at intervals for a period of 4 h. Experiments were repeated with phosphate buffer, pH 5.5 and acetate buffer at pH 5.5; in each case HEWL was tested for activity in the appropriate buffer system.

5.3. Results

5.3.1. Bacterial agglutination

Table 5.1 gives a list of bacteria tested, together with the agglutination titres. It can be seen that of the ten bacteria tested, only three (*P.immobilis*, BS 66 and BS 68) gave a positive agglutination response. Haemocyte lysate supernatants gave a very variable reaction with both *P. immobilis* and BS 68. Table 5.1 shows that a maximum titre of 16 was observed for agglutinating activity for both these bacterial species but in some instances no agglutination was present at all. This occurred with three out of the eight HLS samples tested, and those samples that gave a negative response with *P. immobilis* also gave a negative response for BS 68. For BS 66, all eight samples tested gave a positive response between 2 and 8. There was no difference in titre of agglutination, where present, between microtitre plates incubated at 20 °C or 4 °C.

5.3.2. Removal of agglutinination by adsorption with LPS

In all three experiments, agglutinating activity against *P. immobilis* was completely removed after incubation of HLS with LPS at a final concentration of 1 mg ml^{-1} (Table 5.2). Lipopolysaccharide at a final concentration of 0.1 mg ml^{-1} reduced the agglutination titre to 2, whereas untreated HLS gave a titre of 8 in all cases (Table 5.2.).

5.3.3. Removal of antibacterial activity by adsorption with LPS and bacteria

Table 5.3 shows that pretreatment of HLS with LPS at a final concentration of 1 mg ml^{-1} not only effectively removed antibacterial activity (mean survival index (SI) for *P. immobilis* was 283.8 ± 42.9), but allowed enhanced bacterial growth when

Table 5.1. Bacterial agglutination with HLS[1]

Bacteria[2]	Gram reaction	Titre[6] range	n[7]
Psychrobacter immobilis[3]	-ve	0-16	8
Photobacterium phosphoreum	-ve	0	5
Vibrio anguillarum (serotype I)	-ve	0	5
Vibrio anguillarum (serotype II)	-ve	0	5
Pseudomonas 3-1-1	-ve	0	5
Aerococcus viridans (var.) *homari*[4] Avirulent	+ve	0	5
Aerococcus viridans (var.) *homari*[4] Virulent	+ve	0	5
Planococcus citreus	+ve	0	5
BS 66[5]	+ve	0-16	8
BS 68[5]	+ve	2-8	8

[1]HLS: Haemocyte lysate supernatant, prepared as described in Materials and Methods, Chapter 2, page 60. Protein levels in HLS were standardized to 1.2 mg ml^{-1}.

[2]Bacteria: Prepared in sterile 3.2 % NaCl to a concentration of 10^9 bacteria ml^{-1}.

[3]*Psychrobacter immobilis*, formerly *Moraxella* sp.

[4]*Aerococcus viridans* (var.) *homari*, formerly *Gaffkya homari*.

[5]BS 66 & BS 68: Marine Antarctic species.

[6]Titre is given as the reciprocal of the highest dilution at which agglutination could be recorded.

[7]n: number of HLS samples tested.

Table 5.2. Agglutinating activity against *Psychrobacter immobilis* after pretreatment of HLS[1] with LPS[2]

Treatment	Titre[5]
HLS untreated	8
LPS (1.0 mg ml^{-1})[3]	0
LPS (0.1 mg ml^{-1})[3]	2
Control[4]	0

[1]HLS: Haemocyte lysate supernatant, prepared as described in Materials and Methods, Chapter 2 page 60.

[2]LPS: Lipopolysaccharide from *E. coli* 0111: B4, phenolic extraction (Sigma. Poole, Dorset.)

[3]LPS final concentrations, incubated with HLS for 30 min as described in Materials and Methods, page 126.

[4]Control: 100 μl bacteria and 100 μl modified *Carcinus* saline (CS II).

n = 3

Table 5.3. Antibacterial activity against *Psychrobacter immobilis* after pretreatment of HLS[1] with LPS[2]

Sample	SI[6] (4 h)
HLS-untreated	0.002 ± 0.001
HLS-LPS treated[3]	283.8 ± 42.9
HLS-bacteria treated[4]	15.2 ± 3.4
Control[5]	130.4 ± 5.5

[1]HLS: Haemocyte lysate supernatant, prepared as described in Materials and Methods, Chapter 2, page 60.

[2]LPS: Lipopolysaccharide from *E. coli* 0111: B4 (Sigma. Poole, Dorset.) at a final concentration of 1 mg ml^{-1}.

[3]HLS: Pretreated with LPS for 30 min as described in Materials and Methods, page 130.

[4]HLS: Pretreated with *Psychrobacter immobilis* at a concentration of 5 x 10^9 as described in Materials and Methods, page 130.

[5]Control: Comprised equal parts of modified *Carcinus* saline (CS II) and marine broth as described in Materials and Methods, Chapter 2, page 63.

[6]SI: Survival index calculated as described in Materials and Methods, Chapter 2, page 63. High SI values indicate growth, low SI values indicate antibacterial activity.

n = 3

compared with controls, which had a 4 h SI of 130 ± 5.5. The mean survival index value (SI) for untreated HLS was 0.002 ± 0.001. Preincubation of HLS with *P. immobilis* also eliminated some of the antibacterial activity in HLS, but was not as effective as LPS (Table 5.3). Contamination controls for LPS and bacteria-treated samples were negative.

5.3.4. Lytic activity in HLS

Bacterial lawn assays for the demonstration of growth inhibition or lysis were unsuccessful. In experimental wells, prophenoloxidase in HLS was activated and resulted in blackening of the agar due to deposition of melanin. This masked any lytic or growth inhibitory effect which might have been present (Figure 5.1).

Turbidity measurements at 570 nm were also subject to the same problems of gradual activation of HLS in experimental tubes. Additional controls comprising 500 μl HLS and 500 μl 3.2 % NaCl were set up to monitor for absorbance changes in HLS alone, but this did not compensate for the effect of the bacteria, which caused stronger activation of HLS. As a consequence of this, absorbances at 570 nm for experimental tubes increased rapidly over the experimental period and no evidence of lytic activity against *P. immobilis* could be recorded.

5.3.5. Lysozyme activity in HLS

The salt-supplemented phosphate buffer was compatible with lysozyme activity as shown in Figure 5.2. At the end of the 15 min incubation period, activity was recorded at 50, 10, 5 and 2.5 μg ml^{-1} concentrations of HEWL but not at 0.25 and 0.125 μg ml^{-1}. Maximum activity was recorded for HEWL at 50 μg ml^{-1} with a value of 83.1 % over the controls (Figure 5.2). The standard curve for activity after 15 min incubation is shown in Figure 5.3.

Figure 5.1

Figure 5.1. Melanin deposition around experimental wells (E) in bacterial lawns, after 4 h. Well C is the control, containing modified *Carcinus* saline. Lawns were prepared as described in Materials and Methods, page 126.

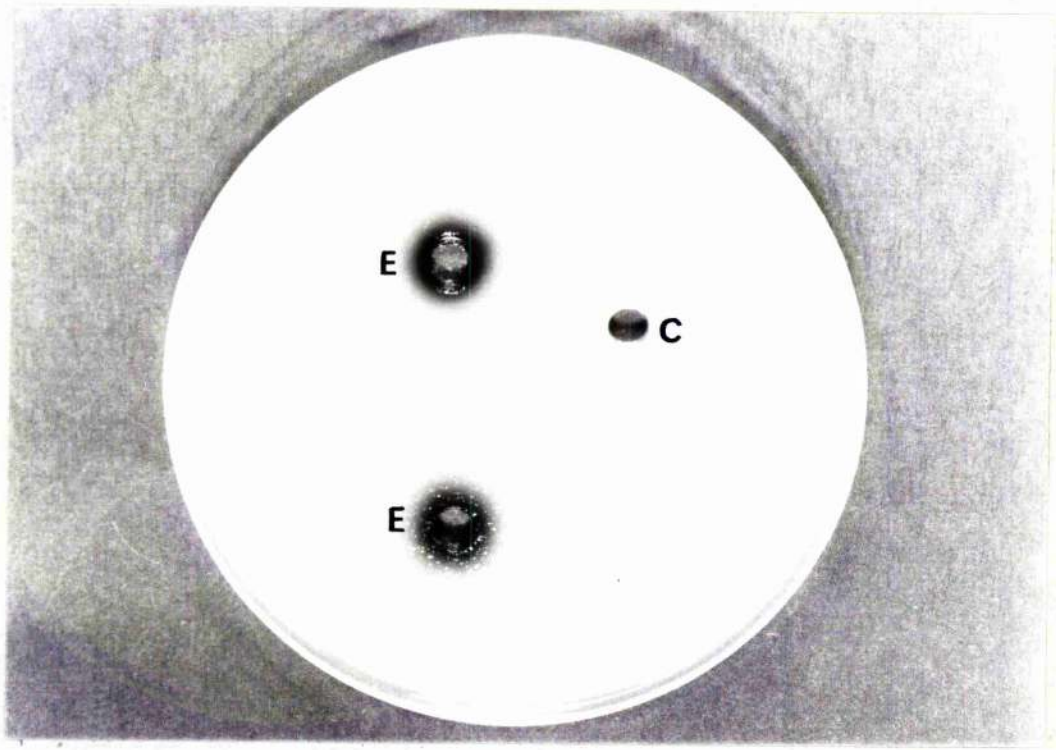
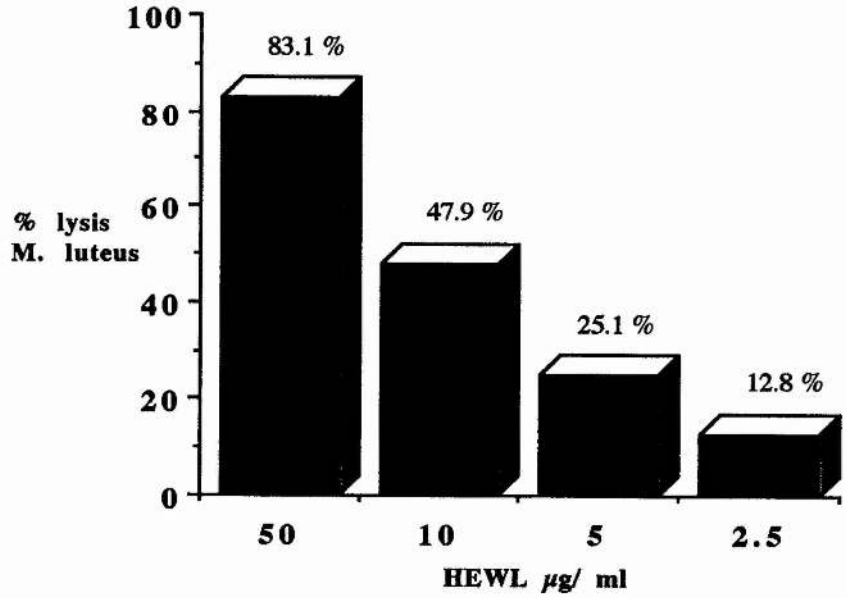
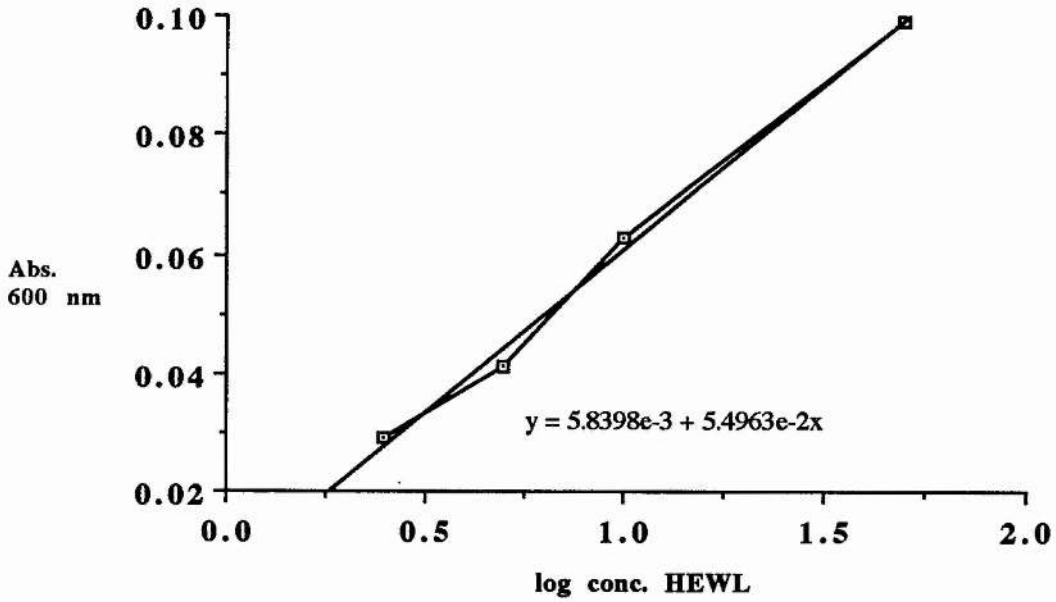

E
C
E

Figure 5.2 & 5.3

Figure 5.2. Activity of different concentrations hen egg-white lysozyme (HEWL) in salt supplemented phosphate buffer, pH 6.4, after incubation with a suspension of *Micrococcus luteus* cell walls at 30 °C for 15 min. Lysis is expressed as a percentage of the control for each concentration of HEWL tested. The experimental method is given in Materials and Methods, page 127.

Figure 5.3. Standard curve for hen egg-white lysozyme (HEWL) after 15 min incubation with a suspension of *Micrococcus luteus* cell walls. HEWL and *M. luteus* were dissolved in a 0.06 M phosphate buffer, pH 6.4, supplemented with 100 mM NaCl as described in Materials and Methods, page 127. Concentrations of HEWL were 50, 10, 5 & 2.5 μg ml^{-1}.


100
80
60
40
20
0
% lysis
M. luteus
83.1 %
47.9 %
25.1 %
12.8 %
50
10
5
2.5
HEWL µg/ ml


0.10
0.08
0.06
0.04
0.02
Abs.
600 nm
y = 5.8398e-3 + 5.4963e-2x
0.0
0.5
1.0
1.5
2.0
log conc. HEWL

Figures 5.4 & 5.5 give the results of typical lysozyme assays using HLS and HyLS. It can be seen that, for HLS (Figure 5.4), after 15 min and 30 min, low levels of lysis were recorded (5.6 % and 6.4 % respectively) which increased to 14.2 % after 1 hour. Thereafter, lytic activity shows an apparent reduction relative to control tubes. Since contamination controls were negative for experimental samples, this observation is probably due, once again, to slow activation of phenoloxidase in experimental tubes and concomitant effects on absorbance readings. The 15 min activity of HLS was equivalent to that given by 1.9 μg HEWL, as calculated from the standard curve in Figure 5.3. No differences were noted when using buffer systems at pH 5.5. The trend was always that of early low levels of lysis, followed by an apparent reduction.

Figure 5.5 gives the results for an experiment that used HyLS instead of HLS. As it can be seen, the results are similar to HLS for the first hour but thereafter lytic actvity shows a small increase, to 18 0 %, which is maintained for the duration of the incubation time.

5.4. Discussion

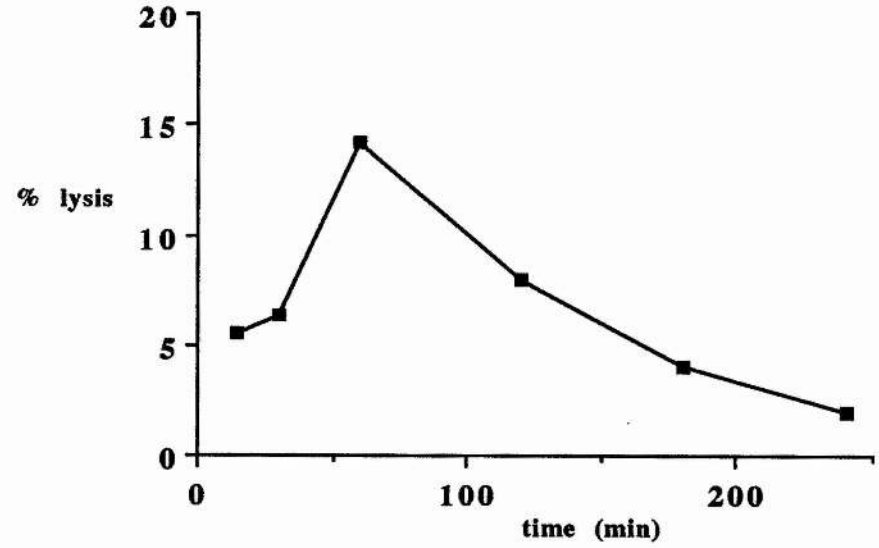
Low levels of bacterial agglutination were recorded for three out of ten species tested. One, *P. immobilis,* was a Gram-negative temperate water species and the other two, BS 66 and BS 68, were Gram-positive Antarctic species. For the rest (three Gram-positive and four Gram-negative species) results were consistantly negative. Other crustaceans have shown similarly low titres of bacterial agglutination (Cornick & Stewart, 1968a,b, 1975; Miller *et al.,* 1972; Huang *et al.,* 1981) with a maximum titre of only 16, when present. It is interesting to note that, in those crustaceans studied so far, agglutination of the lobster pathogen *Aerococcus viridans* (var.) *homari* is the exception rather than the rule. Haemocyte lysate supernatants from *C.*

Figure 5.4 & 5.5

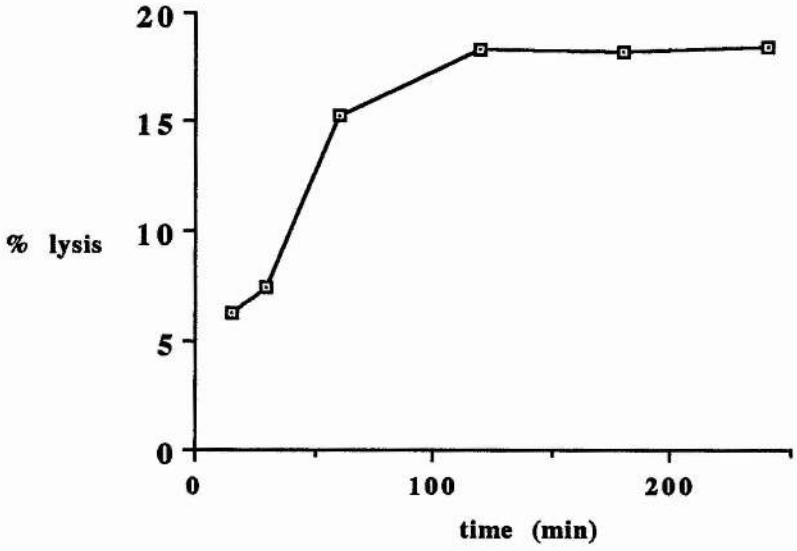
Figure 5.4. Lysis of *Micrococcus luteus* cell walls by haemocyte lysate supernatant (HLS) prepared in 0.06 M phosphate buffer, pH 6.4, as described in Materials and Methods, page 127. Lysis is measured from absorbances read at 570 nm and is expressed as a percentage of the control value. Data relate to a typical experiment. Repeat experiments showed the same trend.

Figure 5.5. Lysis of *Micrococcus luteus* cell walls by hyaline cell lysate supernatant (HyLS) prepared in 0.06 M phosphate buffer, pH 6.4, as described in Materials and Methods, page 127. Lysis is measured from absorbances read at 570 nm and is expressed as a percentage of the control value. Data relate to a typical experiment. Repeat experiments showed the same trend.

HLS


20
15
% lysis
10
5
0
0
100
200
time (min)

HyLS


20
15
% lysis
10
5
0
0
100
200
time (min)

maenas failed to agglutinate *A. viridans* in the work described here. Similarly, Huang *et al.* (1981) and Cornick & Stewart (1968a, 1975) found equivalent lack of actvity in *Macrobrachium rosenbergii, Homarus americanus* and *Geryon quinquedens* respectively. *Chionoecetes opilio,* however, does agglutinate *A. viridans* (Cornick & Stewart, 1975) and so does *Cancer irroratus,* albeit weakly (Cornick & Stewart, 1968b).

Whether the observations reported here were due to true agglutination is debatable. Agglutination, as a classic serological reaction, was first described by Gruber & Durham in 1896 (cited in Wistreich & Lechtman, 1980) and involves the aggregation of antigens (e.g. bacteria or erythrocytes) by homologous antibodies. Agglutination of *P. immobilis* could be abolished by preincubation of HLS with LPS, which might mean that the so-called agglutination reaction was in reality a clotting response. It is known that HLS can undergo clotting independently of the plasma factors (which normally contribute to the clotting of whole haemolymph in macruran crustaceans), providing that protease activity has been induced by the appropriate non-self molecules, such as LPS (Söderhäll, 1981; Durliat, 1985; Durliat & Vranckx, 1989) . However, in the event that the aggregation of *P. immobilis* is due to clotting rather than agglutination, it might be expected that all the Gram-negative bacteria, with their characteristic outer layer of LPS, would elicit the same response, but this was not the case. Cross absorption tests were not carried out here, but could perhaps reveal whether the agglutinating activity for the three species *P. immobilis,* BS 66 and BS 68, is specific or non-specific. In any event, whether it be true agglutination or clotting, the titres of activity were low (maximum of 16, at a protein concentration of 0.08 mg ml^{-1}) compared with the titre of antibacterial activity recorded in Chapter 4, where HLS showed a measurable antimicrobial effect at a dilution of 10^{-4} and a protein concentration of *circa* 2 μg protein ml^{-1}. Thus, it is

unlikely that the clotting/ agglutinating reaction recorded here contributes significantly to the overall observed antibacterial effect in HLS.

Antibacterial activity could also be removed from HLS by preincubation of HLS with 1 mg ml^{-1} LPS, suggesting that for Gram-negative organisms at least, the antibacterial factor or factors bind to the surface of bacteria. As might be expected with antibacterial activity as strong as that found in *C. maenas* HLS, preincubation of HLS with bacteria, even at a concentration of 5×10^9, did not completely eliminate antibacterial activity, although some reduction was seen. One milligram of LPS probably represents considerably more than 5×10^9 bacterial cell walls, which would account for the greater effectiveness of LPS in eliminating antibacterial activity.

The presence of phenoloxidase activity was a major problem when attempting to identify the way in which the antibacterial activity actually immoblises the bacteria. Activation of the proPO system by Gram-negative organisms was to be expected, given that extracts of gram-negative bacteria such as *E. coli* are routinely used as elicitors of the system (Söderhäll & Häll, 1984; Ashida *et al.*, 1974). However it is clear that any lytic activity that may be present does not occur rapidly enough and is not powerful enough to override the spectrophotometric effects of phenoloxidase activation. Blackening of agar plates and darkening of experimental tubes in turbidity assays obscured any growth inhibition or lysis that might have been occurring.

For lysozyme assays, the problem of phenoloxidase activation in HLS was still in evidence, but not to the same degree. After 15 min the amount of lytic activity recorded in experimental mixtures was equivalent to less than that afforded by 1.9 μg ml^{-1} HEWL on the same standard suspension of *M. luteus* cell walls. Lysis was noted in experimental tubes up to 1h for HLS although, thereafter, gradual darkening of mixtures obscured any lytic effects. It was interesting to note that lysozyme-like activity was also present at a low level in HyLS. The absence of phenoloxidase in

these preparations made it easier to monitor events in the microtitre plates. *Micrococcus luteus* is a Gram-positive bacterium and it may be that the cell walls do not activate the proPO system as effectively as LPS and ß, 1-3, glucans.

Since the hyaline cells are the dominant phagocytic cells in *C. maenas*, it might be expected that stronger lytic activity would be present in HyLS. In fact, only low levels of activity were present under the experimental conditions, which was surprising, since phagocytic cells characteristically have plentiful lysosomal enzymes which serve to degrade ingested material (Castle, 1984). There are several possible explanations for this. Firstly, lysozyme or lysozyme-like enzymes may be absent from the crab system; lysozyme degrades cell walls from Gram-positive bacteria, whilst the majority of marine bacteria are Gram-negative. Thus it could be argued that lysozyme would confer no advantage on a crab in terms of evolutionary fitness, since the principal bacterial challenges are likely to be from Gram-negative organisms rather than gram-positive. Another possible explanation is that the intracellular ionic composition of crab haemocytes is not suitable for lysozyme, although this seems unlikely when one considers that lysozyme functions well in other marine animals, such as molluscs (Mc Henery *et al.*, 1986), and in the *in vitro* experiments described in this chapter, the high salt buffer did not prevent HEWL from lysing *M. luteus* cell walls. The possibility that there are enzyme inhibitors in the cell homogenates should not be ignored, nor should the possibility that lysozyme activity in the shore crab could be inducible rather than constitutive. A number of physiological parameters in crustaceans are influenced by diurnal and tidal rhythms, but to what degree this might affect the activity of hydrolytic enzymes in haemocytes is not known. Also, the experimental conditions may not have been optimal for any lysozyme that was present in the haemocytes; lysozyme from different sources has different optimal requirements (see Cheng & Rodrick, 1974; Mc Henery & Birkbeck, 1982).

It was felt that , since the level of lytic activity recorded under these experimental conditions was low, and suffered the additional complication of proPO activation, the results did not merit further investigation into the presence of lysozyme at this stage. There are a number of criteria to be satisfied before a claim for lysozyme activity can be justified; for example, lysozyme is stable in acidic media, loses activity when the temperature in alkaline pH is increased and shows temperature-dependent kinetics (Jolles & Zuili, 1960). A better approach to this problem would be to try to isolate the antibacterial factors in HLS and then examine the characteristics of each bioactive fraction.

In summary, agglutination in HLS, when present, occurred at low titre and did not affect the same range of bacteria as were affected by the antibacterial activity. Lytic activity against the Gram-negative bacterium *P. immobilis* could not be determined due to interference by phenoloxidase activity, and lytic activity against *M. luteus* cell walls was present in HLS and HyLS, but at low level. Thus it seems that antibacterial activity is a genuine phenomenon, independent of agglutination and not entirely due to lysozyme-like activity, although it is possible that these two events contribute in part to the overall effect.

Chapter 6
Preliminary Biochemical Characterization of the Antibacterial Factors in *Carcinus maenas* Haemocytes

6.1. Introduction

A number of characteristics of the antibacterial factor in *Carcinus maenas* have now been established (see Chapters 2-4). However, whether the antimicrobial effect is due to one or several biologically active molecules remains unknown, and the biochemical nature of the activity has yet to be ascertained.

For invertebrates as a group, there has been identification and characterization of several antibacterial molecules. They are found in animals as disparate as sponges and tunicates and, as observed in Chapter 1, may function as general disinfectants, as with echinochrome A in echinoderms (Service & Wardlaw, 1985). Alternatively, they may be highly specific in action, as is lysozyme, which cleaves ß glycosidic links in bacterial cell walls and has been extensively investigated in molluscs (see review by Ratcliffe *et al.*, 1985). Insects in particular have been shown to generate a host of inducible antibacterial peptides, including cecropins, as well as larger inducible molecules, such as attacins. Cecropins are lytic and operate against Gram-positive and Gram-negative bacteria, whereas attacins affect membrane permeability and render bacteria susceptible to the subsequent attack by the low molecular weight cecropins (see reviews by Boman, 1986, 1991).

So far, the investigations described in this thesis have revealed no evidence of lysozyme or lysozyme-like activity, although activation of phenoloxidase in haemocyte lysate supernatants (HLS) may have obscured lytic effects (see Chapter 5). The aim of the work presented in this chapter was to determine whether activity may be ascribed to one or more factors of different molecular size and also to determine whether antibacterial activity in the shore crab is lytic or non-lytic in nature.

Since the biochemical properties of the bioactive antibacterial molecules in HLS were unknown, gel permeation chromatography (GPC), which separates molecules accordng to size, was the technique of choice for attempting to isolate active factors.

Gel permeation chromatography (otherwise known as gel filtration) has the added advantage that, as a rule, the composition of the eluent is unimportant and can be selected for convenience with respect to the general requirements of the separation (Harris & Angal, 1992).

In this chapter, several procedures are described as preliminary investigations. A number of problems were encountered when working with HLS and much of the early experimental work was related to overcoming early difficulties and establishing a satisfactory protocol in which biological activity was retained during the different stages of the investigation. A microtitre plate assay was developed for looking at the antibacterial properties of resultant fractions and was more rapid and economical of materials than the spread plate technique used in previous experiments.

6.2. Materials and Methods

6.2.1. Preliminary investigations

HLS samples

Unless otherwise stated, HLS was prepared as described in Materials and Methods in Chapter 2, page 60. Samples were prepared in 10-12 ml batches, which supplied enough material to perform repeat experiments and obtain reproducibility. Protein levels were 1.5-1.8 mg ml^{-1} in fresh HLS, which was tested for antibacterial activity against *Psychrobacter immobilis* by the spread plate method (see Materials and Methods, Chapter 2, page 63). One millilitre aliquots were stored in sterile Eppendorfs at -20 °C until required. Thawed samples were centrifuged in a microfuge at 14,000 g to ensure that samples were completely free of particulate matter prior to use on columns.

Ammonium sulphate precipitation

The high salt content of routinely prepared HLS samples made them unsuitable for chromatography, where the resultant fractions were to be freeze-dried and then reconstituted in small volumes of buffer for antibacterial assays. It was necessary, therefore, to desalt samples at the outset.

One hundred percent ammonium sulphate precipitation was used as an initial step, and was carried out over a period of 1 h in a cold room at 7 °C. This was facilitated by dilution of 2.0 ml HLS with sterile deionized water to a volume of 20 ml. Addition of ammonium sulphate (14.14 g per 20 ml sample) was done slowly and with continuous stirring. The precipitate was recovered by centrifugation at 3,000 g for 1 h at 4 °C.

The precipitate was redissolved in 2.0 ml sterile modified *Carcinus* saline (CS II, pH 7.4) and used in antibacterial assays against *Psychrobacter immobilis* to check that activity had not been lost during precipitation. Difficulties were encountered in setting up the control for this experiment because the amount of residual ammonium sulphate in the precipitate could not be determined precisely and had to be estimated. The volume of ammonium sulphate solution present in the precipitate, after centrifugation and removal of supernatant, was estimated to be one third of the total precipitate volume. Ammonium sulphate was added to control mixtures of marine broth and CS II in accordance with the final estimated concentration in the experimental tubes. Assays were performed at 20 °C with a 4 h incubation period and contamination controls were included to check for sterility of resuspended precipitates. Protein was measured in reconstituted samples, as described in Chapter 2, page 64.

Desalting with a PD-10 column

As an alternative to ammonium sulphate precipitation, samples were desalted on PD-10 columns containing Sephadex G-25 (Pharmacia. Uppsala,Sweden). In this method only very small molecules enter the solid phase of the gel, which comprises cross-linked dextran beads with fine pore size. Small molecules which have diffused into the gel bed are delayed, whilst larger molecules move continuously down the column and are eluted rapidly. Thus, proteins may be readily partitioned from salts, since there is a large difference in the molecular weights of the components to be separated (Porath & Flodin, 1959).

One millilitre of HLS was applied to a PD-10 column which had been equilibrated with 20 ml sterile deionized water. The sample was eluted at a rate of approximately 1 ml min^{-1} with sterile deionized water. One millilitre fractions were collected by hand and monitored for protein at 280 nm. Fractions containing protein were pooled, freeze-dried and stored at -20 °C until use.

To ascertain whether antibacterial activity had been retained after desalting, the pooled, freeze-dried material was reconstituted in 1 ml sterile CS II and assayed for antibacterial activity against *P. immobilis,* using the spread plate method. Protein was also measured in reconstituted samples as described previously.

Ultrafiltration

Ultrafiltration, using Millipore Ultrafree CL units, was carried out to obtain a preliminary idea of the molecular size range of the antibacterial factor(s) in HLS. Ultrafiltration may also be used as a means of desalting, since small molecules pass into the filtrate, leaving the residue as a desalted sample.

Two millilitres of HLS (protein level adjusted to 1 mg ml^{-1} as recommended) were centrifuged in an Ultrafree unit in a fixed angle rotor at 2,000 g for 2 h at 4 °C.

Ultrafree units with two molecular weight limits (MWL) were used - 5,000 Da and 10,000 Da. Filtrates and residues were used for antibacterial assays against *P. immobilis*. Because of the desalting effect that occurs during ultrafiltration, the filtrate was reconstituted to 2 ml with sterile water whilst the residue was reconstituted to 2ml with CS II. Both fractions were then tested for antibacterial activity by the spread plate method. Controls for filtrates and controls for residues were set up to allow for the greater protein content of the latter. For residues, controls contained equal parts of marine broth and CS II, whereas for filtrates, controls contained only CS II as culture medium. Assays were carried out at 20 °C with a 4 h incubation period.

Inhibition of phenoloxidase activity

Early gel filtration experiments carried out with desalted HLS samples showed that phenoloxidase activation occurred during chromatography and resulted in contamination and irreversible blockage of the column with small particles of melanin. To overcome this problem, phenylthiourea (PTU), a copper binding compound known to inhibit phenoloxidase in insects (Sugumaran *et al.*, 1988), was incorporated into the eluent at a final concentration of 0.01 %, as described in Hultmark *et al.* (1983).

Prior to use in columns, PTU was tested for phenoloxidase inhibition using the protocol described in Chapter 2, page 52. Haemocyte lysate supernatant was preincubated with PTU to a final concentration of 0.01 % for 20 min before addition of elicitor and substrate. Haemocyte lysate supernatant was also assayed for phenoloxidase activity in the absence of PTU, together with a control in which elicitor had been replaced with CS II. Additionally, PTU was tested for effects on bacterial growth, as described below.

Microtitre plate assay for bacterial growth in HLS fractions

Fifty microlitres of bacterial suspension in sterile 3.2 % NaCl, pH 7.0, (prepared to an initial absorbance of approximately 0.2 at 570 nm) were incubated with 50 μl of test sample in round-bottomed microtitre plates. Two sets of controls were set up; one contained 50 μl CS II as a substitute for the test sample and the other (designed to monitor any change of turbidity due to the sample itself) comprised 50 μl 3.2 % NaCl and 50 μl test sample. Plates were incubated at 20 °C and bacterial growth was monitored at 1 h intervals for a period of 6 h. Changes in turbidity were measured on a microtitre plate reader (Dynatech) at 570 nm. Growth curves were produced by plotting absorbance at 570 nm against time.

Bacterial growth in the presence of PTU

PTU was tested for effects on bacterial growth, essentially using the microtitre plate assay described above. In brief, 45 μl bacterial suspension were incubated with 45 μl marine broth and 10 μl of 0.1 % PTU in CS II. Controls contained 10 μl CS II in place of PTU, and plates were monitored for bacterial growth at 570 nm over a period of 4 h. Six replicate wells were used for experimental and control mixtures and the experiment was repeated once.

6.2.2. Gel permeation chromatography (GPC)

Sample preparation

As a result of preliminary investigations, PD-10 columns were routinely used to desalt HLS prior to GPC. Desalted material was freeze-dried and stored at -20 °C until use, when it was reconstituted in 600 μl ammonium acetate (0.1 M, pH 6.5) containing 0.01 % PTU. Protein levels were adjusted to 1.4-1.5 mg ml^{-1}. Before HLS samples were applied to the column they were centrifuged at 4,000 g for 15 minutes at 4 °C to remove any particulate material.

Column preparation and calibration

Sephadex G-100 Superfine, with a fractionation range for globular proteins of 4,000-100,000, was used for GPC. The gel was pre-swollen in eluent (1.5 g Sephadex in 30 ml 0.1 M ammonium acetate, pH 6.5, containing 0.01 % PTU) for 72 h at 20 °C. Excess eluent was removed from the settled gel which was then degassed before pouring the column. Eluent was also degassed prior to use. When the column was not in use it was equilibrated with 0.1 M ammonium acetate containing 0.2 % sodium azide to prevent microbial growth.

A 30 cm column containing Sephadex G-100 gel was set up and connected to a pump (Watson-Marlow, Falmouth, England) and an LKB 2212 HeliRac fraction collector (Pharmacia, Uppsala, Sweden). The gel was packed and equilibrated with 50 ml 0.1 M ammonium acetate, containing 0.01 % PTU, at a flow rate of 6 ml h^{-1}. The flow rate was adjusted to 4 ml h^{-1} for running samples.

For calibration, 150 μl Blue Dextran (1mg ml^{-1} in 0.1 M ammonium acetate) were applied to the column and eluted with 0.1 M ammonium acetate. This provided a check that the gel was evenly packed and also, since Blue Dextran has a very high molecular weight (2,000,000 Da) and is eluted rapidly, determined the void volume (V_o) of the column. The elution volume of Blue Dextran (which is equal to V_o) was recorded and used for calibration purposes. Molecular weight standards comprised albumin (MW 67,00 Da), ovalbumin (MW 43,000 Da), chymotrypsinogen, (MW 25,000 Da) and ribonuclease A (MW 13,700 Da) (Pharmacia, Uppsala, Sweden). Standards were prepared in eluent at a concentration of 5 mg ml^{-1}, and 150 μl samples were applied to the column.

Each standard was run separately, eluted with 0.1 M ammonium acetate (pH 6.5) containing 0.01 % PTU, and elution volumes were recorded for each sample. One millilitre fractions were collected and monitored for protein spectrophotometrically at

280 nm. The elution of a solvent is best characterized by a distribution coefficient, Kd, which represents the fraction of the stationary phase that is available for diffusion of a given solute species (see Preneta, 1992). However, this is difficult to determine in practice and, instead, the coefficient Kav is used. Kav is easier to calculate and there is a linear relationship between Kav and log MW, which is convenient. Kav is calculated by:

$$Kav = \frac{V_e - V_0}{V_t - V_0}$$

V_e = the elution volume of the molecule

V_0 = void volume (which is also equal to the volume of the mobile phase)

V_t = total volume of the packed bed.

A calibration curve (selectivity curve) was prepared by plotting Kav against log MW of the samples and was used to estimate the molecular weights of active fractions following GPC. The theoretical considerations relating to column calibration are found in Preneta (1992).

GPC of HLS

Five hundred microlitres of HLS in 0.1 M ammonium acetate were applied to the column and eluted at 4 ml h^{-1} with 20 ml of 0.1 M ammonium acetate containing 0.01 % PTU. One millilitre fractions were collected by fraction collector and protein was measured spectrophotometrically at 280 nm, using eluent as the blank. Fractions from major protein peaks were freeze-dried overnight to remove the ammonium acetate and stored at -20 °C until required for antibacterial assays.

Antibacterial activity assays

Freeze-dried fractions were reconstituted in 300 μl CS II and used for microtitre plate assays of antibacterial activity against *P. immobilis*. Three replicate experimental wells were set up for each fraction, together with bacterial growth controls and a control for turbidity changes in the sample, as described above. Growth was measured at one hourly intervals for six hours. Individual fractions from each protein peak were tested, and the calibration curve was used to determine approximate molecular weights for active fractions.

6.3. Results

6.3.1 Preliminary investigations

Ammonium sulphate precipitation

Table 6.1 shows that ammonium sulphate in control tubes exerted an antibacterial effect, giving SI values of *circa* 70. Experimental tubes, containing reconstituted precipitate, gave SI values of *circa* 40-50 whereas antibacterial activity in the corresponding fresh HLS had been stronger and gave SI values of *circa* 1.5. Removal of residual ammonium sulphate by dialysis was not attempted since previous experience with this technique had shown that it was impossible to maintain sterility (see Chapter 4, page 118). Protein recovery was less than 70 % following salting out. Initial protein level in HLS was 1.6 mg ml^{-1} but after ammonium sulphate precipitation protein levels had dropped to 1.1 mg ml^{-1} in reconstituted samples.

Salting out with different concentrations of ammonium sulphate is a commonly used technique for the recovery of protein fractions (Harris & Angal, 1992). In this

Table 6.1. Antibacterial activity in HLS[1] after desalting by ammonium sulphate precipitation and PD-10 columns

Sample	SI[2] (HLS)	SI (Control)[3]
Fresh HLS	1.3 ± 0.7	129 ± 20.3
Desalted HLS (ammonium sulphate)[4]	45.3 ± 10.2	70.5 ± 13 .2
Desalted HLS (PD-10)[5]	18.1 ± 4.4	145 ± 15.9

[1]HLS: Haemocyte lysate supernatant, prepared as described in Materials and Methods, Chapter 2, page 60.

[2]SI: Survival index calculated as described in Materials and Methods, Chapter 2, page, 63. Values >100 indicate growth, values < 100 indicate antibacterial activity.

[3]Controls for fresh HLS samples and those desalted with PD-10 columns were set up as described in Materials and Methods, Chapter 2, page 63, with equal parts of modified *Carcinus* saline (CS II) and marine broth as a substitute for HLS. Controls for samples arising from ammonium sulphate precipitation were set up in the same way but also included ammonium sulphate as described in Materials and Methods, page 148.

[4]Desalting by 100 % ammonium sulphate precipitation was carried out as described in Materials and Methods, page 148. Precipitates were reconstituted in modified *Carcinus* saline (CS II) and used to check for retention of antibacterial activity after desalting.

[5]Desalting by means of PD-10 columns was carried out as described in Materials and Methods, page 150. Fractions were pooled, freeze-dried, reconstituted in CS II and used to check for retention of antibacterial activity after desalting.

Values shown are the means ± standard deviations for two repeat experiments, using material from the same batch of HLS.

instance, the sensitivity of *P. immobilis* to ammonium sulphate made the technique unsuitable.

Desalting with a PD-10 column

Protein was eluted in the two major peaks shown in Figure 6.1. In all cases, antibacterial activity was retained after desalting by this method (see Table 6.1), although there was some loss of activity compared with fresh samples, with SI values of *circa* 18 for desalted material compared with 1.5 for fresh HLS. Protein recovery was better than that acheived with ammonium sulphate precipitation (protein 1.3 mg ml^{-1} for PD-10 samples compared with 1.1 mg ml^{-1} from ammonium sulphate precipitation), although there was still some loss compared with fresh material (1.6 mg ml^{-1}).

Ultrafiltration.

Table 6.2 gives the results of antibacterial assays using filtrates and residues after ultrafiltration at molecular weight cut off points of 5,000 Da and 10,000 Da. Strong antibacterial activity was present in residues from both 5,000 and 10,000 molecular weight limit (MWL) units, with little difference between the two, indicating that the MW of bioactive molecules in residues are in excess of 10,000 Da. As regards filtrates, antibacterial activity was present at low level in the filtrate from the 10,000 MWL units (Table 6.2), giving an SI of 60.2 ± 8.4, but not from the 5,000 MWL units (SI of 100.5 ± 6.9). Thus it appeared that antibacterial activity in HLS was probably due to more than one component,with molecular weights in excess of 5,000 Da.

Inhibition of phenoloxidase

It was ascertained that PTU at a concentration of 0.01 % was effective as an inhibitor of phenoloxidase for the purposes of GPC. HLS samples treated with 0.01

Figure 6.1

Figure 6.1. Typical elution profile for desalted haemocyte lysate supernatant (HLS) from a PD-10 column. HLS was prepared as described in Materials and Methods, Chapter 2, page 60, eluted at a flow rate of 1ml min^{-1} and collected as 1 ml fractions. PD-10 column was equilibrated in deionized water containing 0.01 % phenylthiourea (PTU). Fractions 3-10 were pooled and freeze dried and stored at -20°C until use.

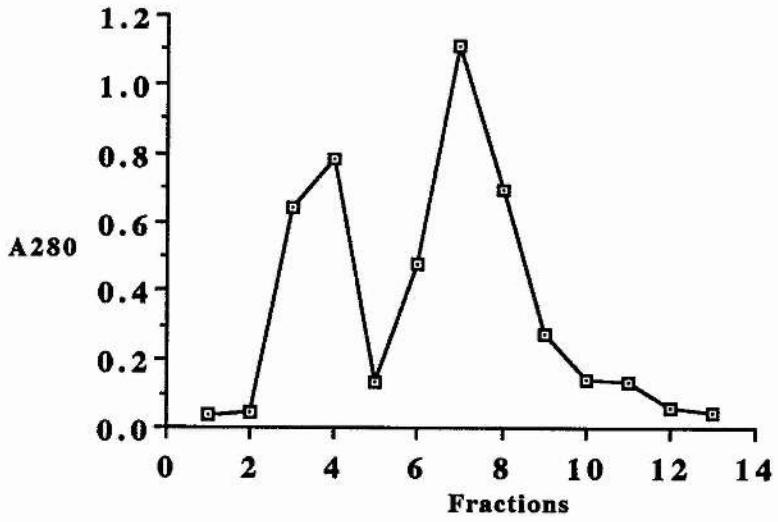

1.2
1.0
0.8
0.6
0.4
0.2
0.0
A280
0
2
4
6
8
10
12
14
Fractions

Table 6.2. Antibacterial activity in HLS[1] after ultrafiltration[2]

HLS Sample	SI[3] (HLS)	SI (Controls)[4]
Filtrate (5,000 MWL)	100.5 ± 6.90	102.40 ± 5.15
Residue (5,000 MWL)	0.09 ± 0.02	128.55 ± 6.44
Filtrate (10,000 MWL)	60.2 ± 8.40	102.40 ± 5.15
Residue (10,000 MWL)	1.30 ± 0.90	128.55 ± 6.44

[1]HLS: Haemocyte lysate supernatant prepared as described in Materials and Methods, Chapter 2, page 60.

[2]Ultrafiltration performed using Millipore Ultrafree CL units as described in Materials and Methods, page, 150.

[3]SI: Survival index calculated as described in Materials and Methods, Chapter 2, page 63. Values > 100 indicate bacterial growth, values < 100 indicate the presence of antibacterial activity.

[4]Controls were set up as described in Materials and Methods, page 151.

[5]MWL: Molecular weight limit of Millipore Ultrafree units.

% PTU and elicited with lipopolysaccharide (LPS) gave phenoloxidase activities of 13.6 ± 3.1 units min^{-1} mg protein $^{-1}$, where one unit of activity is defined as the amount of enzyme activity which gives an increase in absorbance of 0.001 at 490 nm at 20 °C under the experimental conditions. Controls gave 6.5 ± 2.4 units min^{-1} mg protein $^{-1}$, and HLS in the absence of PTU gave 135 ± 16 units min^{-1} mg $protein^{-1}$ (n = 3).

Bacterial growth in the presence of PTU

It was also found that PTU did not act as an antibacterial agent. Figure 6.2 shows a bacterial growth curve for *P. immobilis* incubated in the presence and absence of 0.01 % PTU. It can be seen that, under the conditions of the experiment, PTU did not inhibit bacterial growth, and bacteria grew well in both experimental and control mixtures. Thus, PTU at a final concentration of 0.01 % was routinely added to the deionized water for desalting on the PD-10 column and also to the eluent buffer for column chromatography.

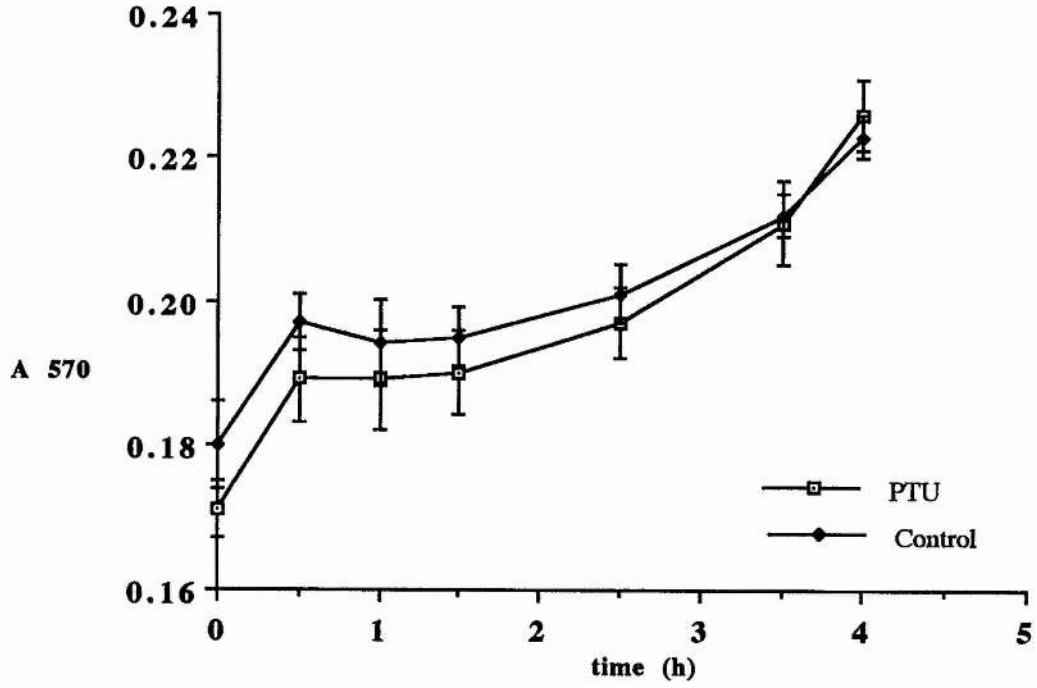
6.3.2. Gel permeation chromatography

Figure 6.3 shows the calibration curve for the column, together with the equation for the line of best fit, which was used to estimate the molecular weight of the active fractions from HLS. Figure 6.4 shows a typical elution profile for gel filtration of HLS in ammonium acetate, and three main protein peaks are present (A, B, & C in Figure 6.4). Fractions from each protein peak were used in bacterial growth assays, and growth curves for *Psychrobacter immobilis* from a typical experiment are shown in Figure 6.5 A-D. Because there were relatively few fractions to assay from each gel filtration run, fractions resulting from a single sample were tested at the same time and against the same growth control. Control wells containing only test sample and 3.2 % NaCl showed an increase in absorbance of 0.002 - 0.003 at 570 nm over the duration of the antibacterial assay.

Figure 6.2 & 6.3

Figure 6.2. Growth of *Psychrobacter immobilis* in the presence of 0.01 % phenylthiourea (PTU). Growth was monitored at 570 nm, using a microtitre plate reader, as described in Materials and Methods, page 151. Modified *Carcinus* saline (CS II) was used as a substitute for PTU solution in control wells. Values are means ± standard deviations for one experiment using six replicates for experimental and control mixtures.

Figure 6.3. Selectivity curve for Sephadex G-100 Superfine, equilibrated in ammonium acetate (0.1 M, pH 6.5) containing 0.01 % phenylthiourea (PTU). Molecular weight standards were albumin (MW 67,000), ovalbumin, (MW 43,000), chymotrypsinogen (MW 25,000) and ribonuclease A (MW 13,700), prepared at a concentration of 5 mg ml^{-1} in ammonium acetate (0.01 M pH 6.5) with 0.01 % PTU. Kav for each standard was calculated from the formula given in Materials and Methods, page 153. A 30 cm x 9 mm column was used at a flow rate of 4 ml h^{-1}.


0.24
0.22
0.20
0.18
0.16
A 570
0
1
2
3
4
5
time (h)
PTU
Control

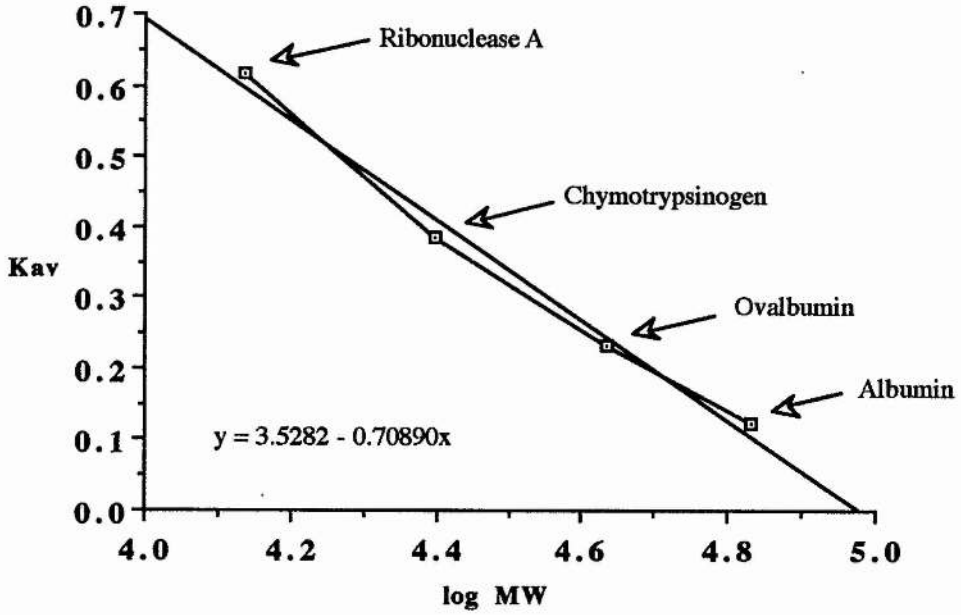
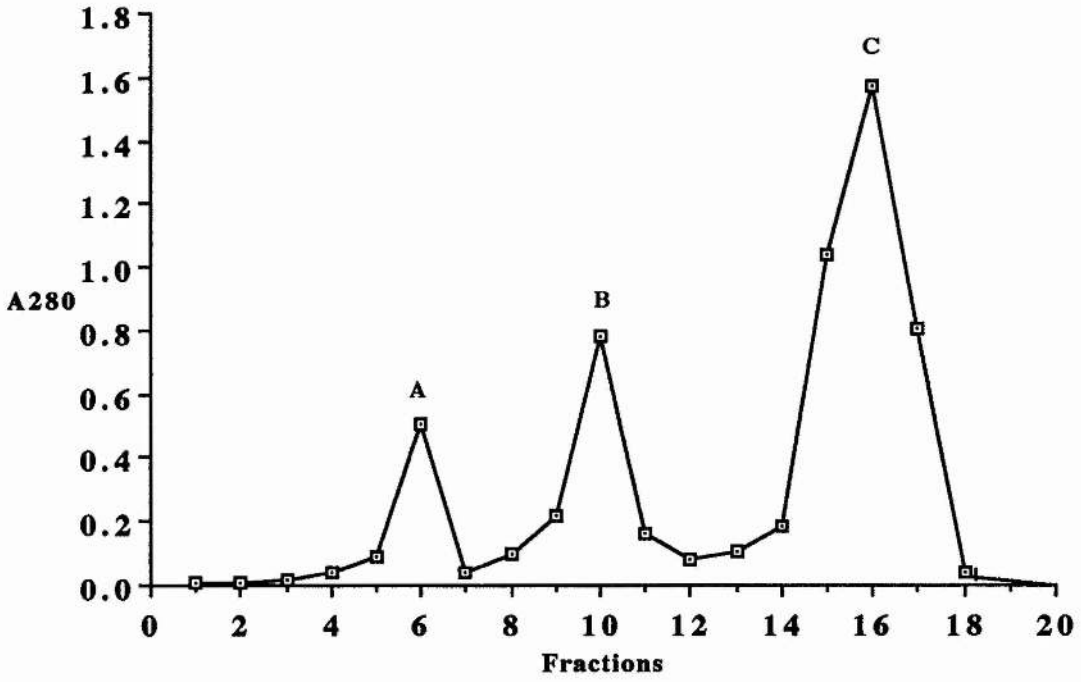

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Kav
4.0
4.2
4.4
4.6
4.8
5.0
log MW
Ribonuclease A
Chymotrypsinogen
Ovalbumin
Albumin
y = 3.5282 - 0.70890x

Figure 6.4

Figure 6.4. Elution profile for haemocyte lysate supernatant (HLS) on Sephadex G-100 Superfine, equilibrated in ammonium acetate (0.1 M, pH 6.5) with 0.01 % phenylthiourea (PTU). HLS was desalted, freeze dried and resuspended in eluent for gel permeation chromatography (GPC), as described in Materials and Methods, page 153. A 30 cm x 9 mm column was used, with a flow rate of 4 ml h^{-1}.


1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A280
A
B
C
0
2
4
6
8
10
12
14
16
18
20
Fractions

From Figure 6.5 A, which relates to fractions from protein peak A, it can be seen that fraction 6 causes inhibition of bacterial growth. During the six hours incubation, the mean absorbance for bacteria incubated with fraction 6 remains unchanged at each time point, whilst bacteria incubated in fraction 5 show steady growth in parallel with the controls. Fraction 6 also contained the most protein from peak A.

Similarly, in Figure 6.5 B (fractions from protein peak B), fraction 9 causes growth inhibition when compared with other fractions from the same peak. However, fraction 9 did not contain the maximum protein for peak B.

Figure 6.5 C & D relate to the fractions from the third protein peak, peak C. Bacterial growth occurs in fractions 13 and 14 during the six hour incubation period, although there appears to be growth inhibition in fraction 13 for the first three hours, and in both fractions the growth pattern is more erratic than in the control. Figure 6.5 D shows that fractions 15, 16 and 17 all cause growth inhibition, and the gradual reduction of turbidity of the experimental wells, particularly for fraction 16, suggests the presence of lytic activity. Protein was maximal in fraction 16.

Kav for fractions 6, 9 and 16 was calculated and the equation for the selectivity curve (Figure 6.3) was used to estimate the respective MW, which were *circa* 72,000 Da (fraction 6), 34,000 Da (fraction 9) and 4,000 Da (fraction 16).

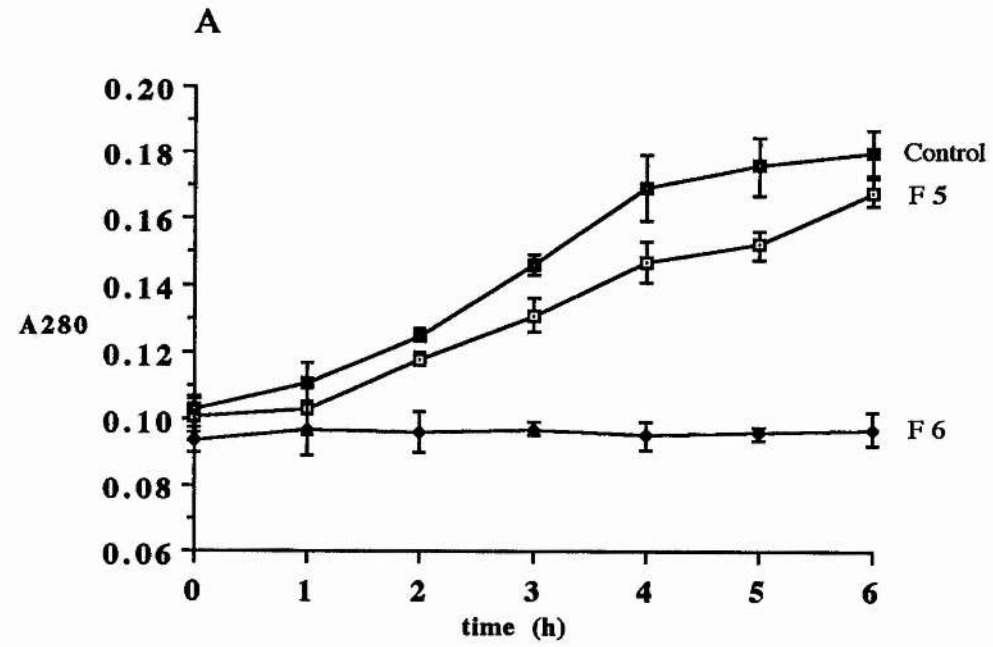
6.4. Discussion

Preliminary analysis of HLS has shown that antibacterial activity in HLS can be attributed to at least three proteins with estimated molecuar weights of 72,000 Da, 34,000 Da and 4,000 Da. The antibacterial effect appeared to be bacteriostatic rather than bacteriolytic for the 72,000 Da and 34,000 Da proteins, since absorbances in experimental wells remained unchanged over the incubation period of the experiment.

Figure 6.5A & B

Figure 6.5 A Growth of *Psychrobacter immobilis* during incubation with fractions from protein peak A (see Figure 6.4). After gel filtration (Materials and Methods, page 153), fractions containing protein were freeze-dried, resuspended in modified *Carcinus* saline (CS II) and used in microtitre plate assays to determine the presence of antibacterial activity as described in Materials and Methods, page 154.

Figure 6.5 B Growth of *Psychrobacter immobilis* during incubation with fractions from protein peak B (see Figure 6.4). After gel filtration (Materials and Methods, page 153), fractions containing protein were freeze-dried, resuspended in modified *Carcinus* saline (CS II) and used in microtitre plate assays to determine the presence of antibacterial activity as described in Materials and Methods, page 154.


A
0.20
0.18
0.16
0.14
A280
0.12
0.10
0.08
0.06
0
1
2
3
4
5
6
time (h)
Control
F 5
F 6

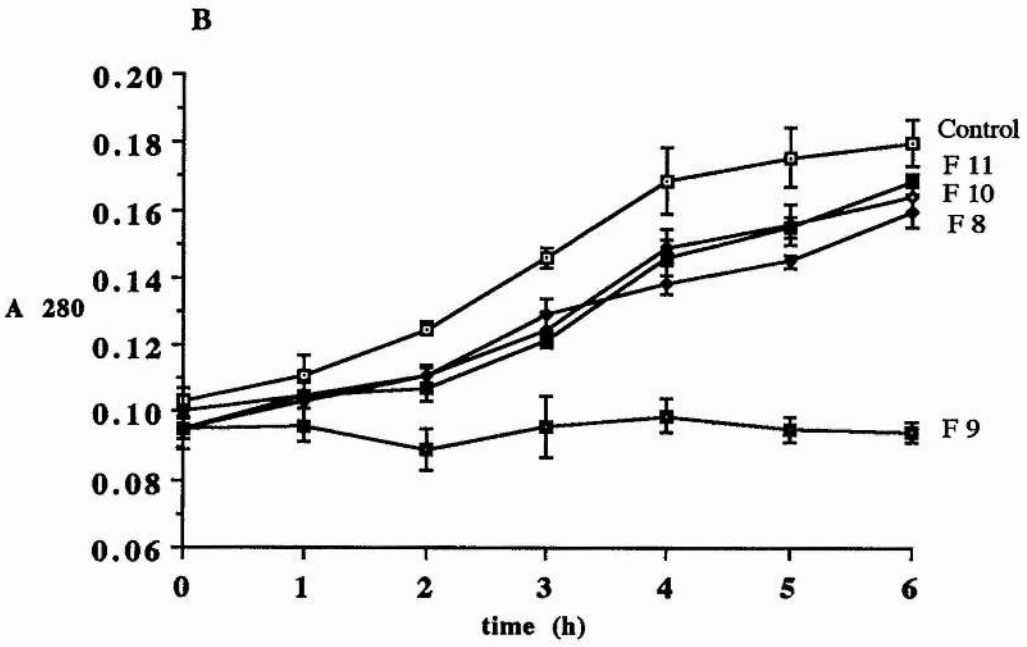
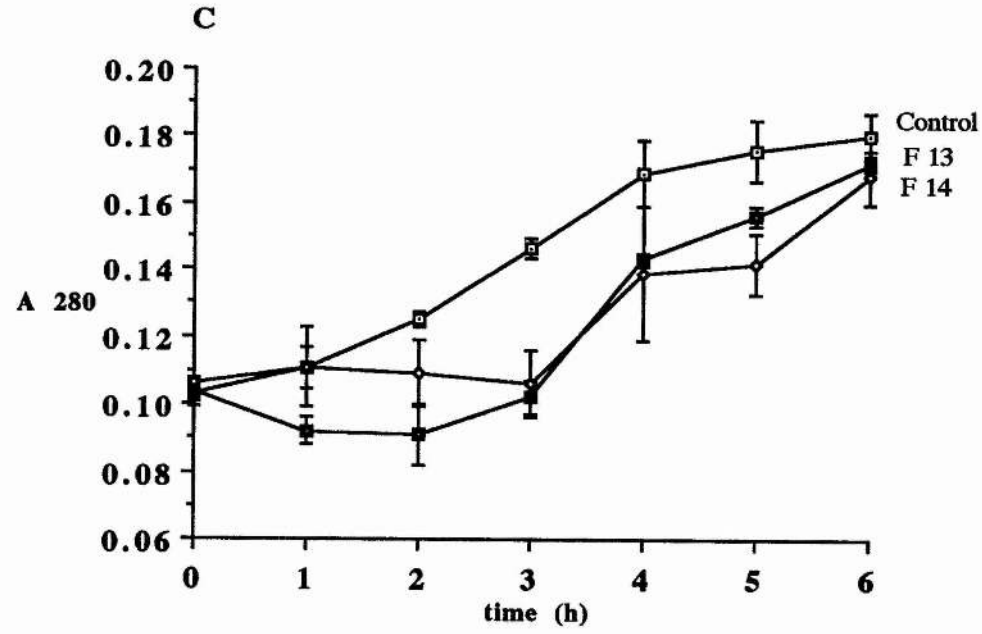
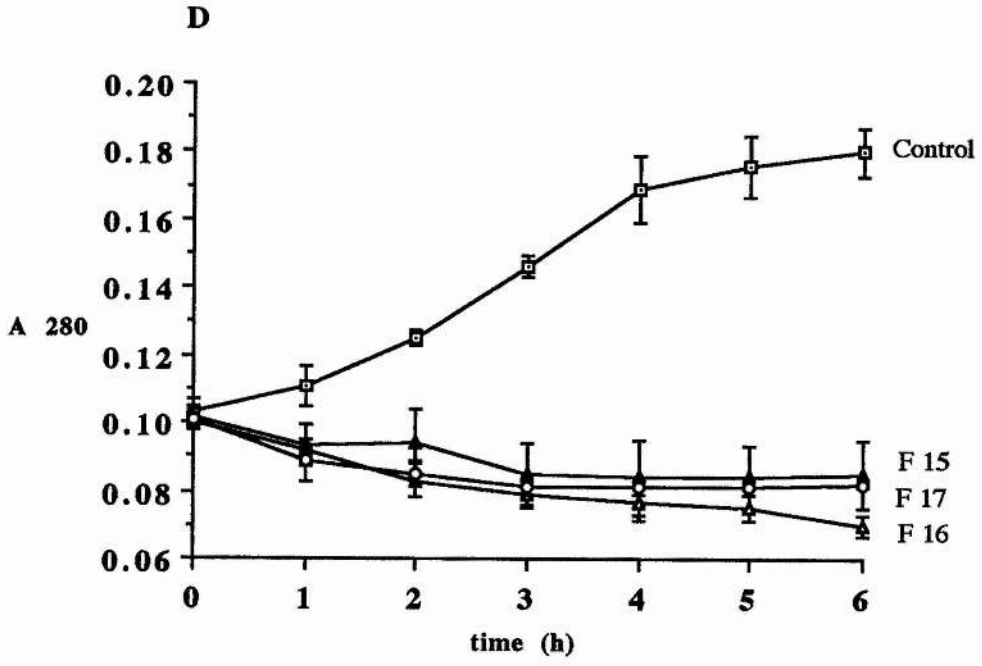

B
0.20
0.18
0.16
0.14
A 280
0.12
0.10
0.08
0.06
0
1
2
3
4
5
6
time (h)
Control
F 11
F 10
F 8
F 9

Figure 6.5C & D

Figure 6.5. C Growth of *Psychrobacter immobilis* during incubation with fractions 13 and 14 from protein peak C (see Figure 6.4). After gel filtration (Materials and Methods, page 153), fractions containing protein were freeze-dried, resuspended in modified *Carcinus* saline (CS II) and used in microtitre plate assays to determine the presence of antibacterial activity as described in Materials and Methods, page 154.

Figure 6.5. D Growth of *Psychrobacter immobilis* during incubation with fractions 15, 16, and 17 from protein peak C (see Figure 6.4). After gel filtration (Materials and Methods, page 153), fractions containing protein were freeze-dried, resuspended in modified *Carcinus* saline (CS II) and used in microtitre plate assays to determine the presence of antibacterial activity as described in Materials and Methods, page 154.


C
0.20
0.18
0.16
0.14
A 280
0.12
0.10
0.08
0.06
0
1
2
3
4
5
6
time (h)
Control
F 13
F 14


D
0.20
0.18
0.16
0.14
A 280
0.12
0.10
0.08
0.06
0
1
2
3
4
5
6
time (h)
Control
F 15
F 17
F 16

Fractions 15, 16 and 17 from protein peak C all showed antibacterial activity and for fraction 16 (4,000 Da) there was a 50 % reduction of turbidity in experimental wells at the end of the 6 h incubation period, which suggested bacteriolysis rather than bacteriostasis.

Sephadex G-100 Superfine was selected for gel filtration because it gave a wide fractionation range of molecular weights (4,000 -100,000) for globular proteins. The superfine grade provides good resolution with low molecular diffusion and therefore less zone broadening (Prenata, 1992).

It must be emphasised that the molecular weights given for fractions 6, 9 & 16 can only be regarded as approximate. Firstly, the conformation of a native protein molecule may affect its behaviour during gel filtration: linear proteins generally appear to have a larger molecular weight than is actually the case when chromatographed in the native state, whereas for globular proteins, gel filtration on a calibrated column of suitable size can provide more accurate estimates of molecular weight (Andrews, 1965). Where preservation of activity is not required, however, gel filtration under denaturing conditions is a useful alternative to electrophoresis as regards molecular weight determinations (Andrews, 1970).

Secondly, Kav values for fractions 6 and 16 fell outside the range given by the calibration proteins albumin (MW 67,000 Da), and ribonuclease A (MW 13,700 Da). For fraction 16, the estimated molecular weight of 4,000 Da lay at the limit of resolution of Sephadex G-100. Nevertheless it would seem that the active molecule in protein peak C is small. The findings from gel filtrations are consistant with the results from ultrafiltration, which suggested that low MW antibacterial molecules (MW <10,000 > 5,000 Da) were present in HLS as well as antibacterial proteins with a MW > 10,000 Da. To identify the molecular weight of the active protein in peak C more accurately, it would be necessary to use Sephadex G-25 or Sephadex G-50,

which are suitable for separations of peptides and other small biomolecules. Alternatively, fractions from protein peak C could be run under denaturing conditions, as mentioned earlier in this discussion.

During gel filtration there is inevitable dilution of the molecules of interest. However, these can be recovered by freeze-drying or evaporation if an appropriate eluent has been used. Thus, volatile eluents (such as ammonium acetate or ammonium carbonate) are lost during freeze-drying, whilst the protein component is recovered. Protein can then be redissolved in buffers appropriate to the next stage of the procedure.

Phenylthiourea (0.01 %), as an inhibitor of phenoloxidase activity, was added to eluents, both at the desalting stage and for GPC with Sephadex G-100. This effectively resolved the problem of melanin formation in the column and, in addition, the presence of PTU did not adversely affect antibacterial assays. It is common practice to incorporate enzyme inhibitors in buffers during GPC in order to reduce loss of activity in native proteins (Benyon, 1992). Loss of activity can also be reduced by carrying out GPC in a cold room. Unfortunately cold room facilities were not available at the time of experimentation and chromatography was carried out at room temperature.

Successful gel filtration requires a number of experimental parameters to be fulfilled. The column used in this work was only 30 cm long, with an internal diameter of 9 mm, which was smaller than is usual for work of this kind. Since the resolution of two separated zones increases with the square root of the column length, long columns are better for analytical resolution, and also allow larger sample sizes to be used. A sample volume of 0.5-5 % of the bed volume is recommended (Prenata, 1992), which, for the column used here, put an upper limit of 900 μl on the sample size. In reality, separation was poor with 900 μl, and 500 μl was finally used for

routine runs. Sample size is also limited by viscosity, since high sample viscosity results in an irregular flow pattern and consequent broadening of the zones. Viscosity was not a problem in the work described here, since sample proteins were usually in the region of 2 mg ml^{-1} and, in practice, a protein concentration of 10-20 mg ml^{-1} is allowable when using an aqueous buffer (Preneta, 1992). As regards running conditions, the lower the flow rate, the better the resolution. Because of the small dimensions of the column used in the work presented here, the flow rate was kept to 4 ml h^{-1}, although (according to Pharmacia recommendations), for a larger column (e.g. 2.5 cm diameter and bed height of 30 cm) flow rates of up to 1 ml min^{-1} may be used for Sephadex G-100 Superfine.

The small sample volumes applied to the column meant that only small amounts of protein were recovered in each fraction. Freeze-dried material was therefore reconstituted in reduced volumes of CS II (300 μl) in order to maximise any antibacterial activity present in fractionated HLS. Protein loss may occur for a variety of reasons at each stage of a purification procedure and clearly it is advantageous to take steps to limit this where possible.

The release of "sticky" proteins from haemocytes has been reported for insects and crustaceans (see Söderhäll & Smith, 1986a,b for reviews). Since proteins in general tend to adhere to foreign surfaces, it is more than likely, in the work presented here, that protein losses occurred during sample storage in Eppendorfs and during transference of samples from one container to another. As far as possible, the number of transfers was kept to a minimum in order to preserve maximum protein.

Protein loss can also accur during chromatography itself. Molecules may bind to the gel, which inevitably reduces the recovery of protein in samples. From the results presented here it was unlikely that significant losses arose in this way: Kav values for eluted fractions should lie between 0 and 1 (Prenata, 1992). If Kav is below zero it

suggests that channelling is present in the gel and the column needs to be repoured. On the other hand, if Kav is greater than 1 it indicates that there has been binding of protein to the gel matrix. In the fractionation of HLS samples from *C. maenas*, all Kav values lay between 0 and 1.

The microtitre plate assay for antibacterial activity was economical of both time and materials: 300 μl of redissolved protein provided adequate material for replicate wells to be set up for each fraction. Previously this type of growth assay had not been possible because phenoloxidase activity, and the inevitable formation of melanin, masked the bacterial responses to test substances. However, the incorporation of 0.01 % PTU in the eluents used for desalting and for GPC largely resolved this problem. Fractions that had been freeze-dried and redissolved in CS II gave negative results in rapid drop assays (see Materials and Methods, Chapter 4, page 103 for protocol), suggesting that phenoloxidase was either inhibited, present in very low concentration or had been substantially deactivated during the various stages of the procedure.

Turbidometric studies have been used rarely to assess antibacterial activity in crustacean haemolymph, which may be a reflection of the difficulties that arise when trying to run an experiment of this kind in the presence of an active phenoloxidase system. However, Fenouil & Roch (1992) have identified the presence of lysozyme in six species of freshwater crayfish in crustaceans using turbidity measurements and do not appear to have encountered this problem. This is probably due to the fact that their haemolymph samples were assayed against cell walls of a Gram-positive bacterium, *Micrococcus luteus* , which may not activate prophenoloxidase to the same extent as the LPS of Gram-negative bacteria such as *P. immobilis.*

The fact that at least three different proteins seem to be involved in the antibacterial activity in *C. maenas* HLS opens up an exciting area for further research. As already mentioned, the haemolymph of cecropid moths contains a battery of antimicrobial

proteins (Boman, 1986) and it may be that crustacean haemolymph is similarly endowed. Given the close phylogenetic relationship of these two arthropod groups, it would not be unreasonable to speculate that this might be the case. The apparent lytic effect of fractions from peak C, which appear to be small molecules, suggests that antimicrobial peptides might occur in *C. maenas*. It has been reported in Chapter 5 that little evidence of lysozyme or lysozyme-like activity could be found, so it was interesting to note that no antibacterial activity was found in protein peaks relating to a molecular weight range of 14,000-15,000 Da, into which lysozyme falls.

With reference to the work carried out on thermal stability of HLS in Chapter 4, measurable residual antibacterial activity was present after treatment at 100 °C for 30 min and it is possible that this could be due to the presence of a heat stable peptide. Peptides have few disulphide bridges, and show less conformational change (and therefore retain more of their activity) when heated, than do larger proteins. The latter have complex tertiary structures (involving disulphide bridges) which are more easily denatured (Stryer, 1981).

Confirmation of molecular weights of active fractions is needed, although within the limitations of the equipment available, gel filtration has proved a useful technique and has provided samples that have retained their activity. Repetition of this work, using a larger column and larger samples would firstly improve the yield of active molecules and, secondly allow pooled fractions from each of the peaks to be further analysed by a variety of techniques with respect to their biochemical properties. This has been successfully carried out for the antimicrobial molecules in insects; for example, Hultmark *et al.*, (1983) have applied the techniques of gel filtration, chromatofocussing and ammonium sulphate precipitation, in combination with antibacterial assays, to identify the several forms of attacins in insects.

Whether the antibacterial molecules in HLS operate independently or synergistically is not known and is a question that awaits further research, and the precise mode of action of each of the factors has yet to be elucidated. There are several mechanisms whereby antibacterial agents can inhibit or kill microorganisms. These include inhibition of metabolism, inhibition of cell wall formation, inhibition of protein synthesis, irreversible damage to the cell membrane and inhibition of nucleic acid synthesis (Wistreich & Lechtman,1980). If the antibacterial factors from *C. maenas* HLS can be purified, it should be possible to determine their mode of action by a range of techniques, including radiolabelling, electron microscopy and molecular probes.

Chapter 7
Antibacterial Activity in HLS from Different Crustacean Species

7.1. Introduction

So far, all the experimental work described in this thesis has related to the shore crab, *Carcinus maenas*. In view of the limited information relating to antibacterial activity in crustaceans in general, it was decided to investigate the antibacterial properties in the haemocytes of four other marine crustaceans, for whom information is lacking.

Three of the selected species were *Galathea strigosa* (squat lobster), *Nephrops norvegicus* (Norway lobster) and *Crangon crangon* (the brown shrimp). These animals are native to British waters, are members of the Order Decapoda and belong to the infraorders Anomura, Astacidae and Caridea respectively (Figures 7.1, 7.2, and 7.3.). They have all been investigated with respect to haemocyte phenoloxidase activity (Smith & Söderhäll, 1991) and, more recently, with regard to *in vitro* superoxide production by phagocytes (Bell & Smith, 1993. Submitted).

The fourth species, *Glyptonotus antarcticus* (Figure 7.4), is a polar invertebrate belonging to the Order Isopoda. Isopods are dominant members in Antarctic benthic marine communities, are scavengers in habit and replace the decapods that are commonly found in warmer waters (Luxmoore, 1982). Compared with their temperate water counterparts, Antarctic isopods show larger body size, slower growth and greater longevity (Luxmoore, 1982). There are no previous studies of immunocompetence in marine polar invertebrates, so the use of *G. antarcticus* as an experimental animal allowed an *in vitro* comparison of antibacterial activity in a polar species with antibacterial activity in species from temperate waters.

Previous work in relation to antibacterial activity in crustaceans has been sparse and has tended to focus on one experimental species at a time. There has been little by

Figures 7.1 &7.2

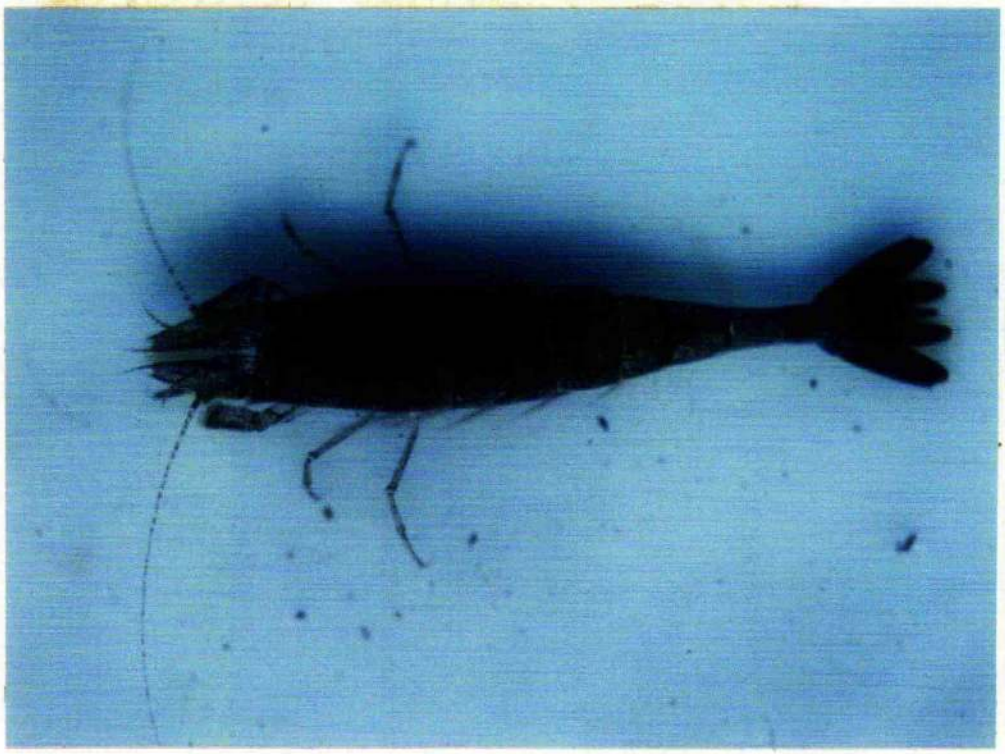
Figure 7.1. *Galathea strigosa,* the squat lobster (Decapoda. Anomura), measuring 6 cm body length

Figure 7.2. *Nephrops norvegicus,* the Norway lobster (Decapoda. Astacidae), measuring 14 cm body length

Figures 7.3 & 7.4

Figure 7.3. *Crangon crangon,* the brown shrimp (Decapoda. Caridea), measuring 5cm body length.

Figure 7.4. *Glyptonotus antarcticus,* the giant Antarctic isopod (Isopoda), measuring 12 cm body length.



way of comparative work, whereby the same experimental protocol is applied to several species, and thus it is difficult to assess the relative effectiveness of the antibacterial responses found in different Crustacea. Here, the aim was to extend the range of crustaceans investigated for antibacterial activity and to compare the antibacterial activities in found in HLS from *C. maenas* with that of *G. strigosa, N. norvegicus, C. crangon* and *G. antarcticus.*

7.2. Materials and Methods

7.2.1. Animals

Galathea strigosa, N. norvegicus and *C. crangon* were collected from St. Andrews Bay in Scotland. Body lengths were 5 - 6 cm, 14 - 15 cm and 6 - 7 cm respectively, and species were maintained in separate tanks in a flowing seawater aquarium (7 °C - 10 °C ± 1 °C) as described for *C. maenas* (Chapter 2, page 56). *Galathea strigosa* and *C. crangon* were usually acclimated for 4-5 days, whereas specimens of *N. norvegicus* were acclimated for at least 7-10 days since they showed high mortality rates in the first week of captivity (approximately 60 %).

Glyptonotus antarcticus, (12- 14 cm body length) were generously provided by the British Antarctic Survey. Animals were kept in recirculating seawater at 0 °C ± 1 °C in a cold room, and fed weekly on live *Crangon.* Specimens were acclimated for 4-5 days before bleeding.

7.2.2. Bleeding procedures

The general principles for obtaining haemocytes, described for *C. maenas* (Chapter 2, page 60), were also adhered to for the above species. Needles and syringes of a size appropriate to the animal were used, and haemolymph was

withdrawn into sterile marine anticoagulant (MAC), pH 4.6, to avoid clotting. For all animals, blood was diluted 1:1 with MAC. *Galathea strigosa, N. norvegicus* and *G. antarcticus* were bled from the unsclerotized membrane at the base of the last walking leg, where a generous pool of haemolymph was usually located. Haemolymph yields were 1.5 ml, 2 ml and 1 ml for *G. strigosa, N. norvegicus* and *G. antarcticus* respectively. *Crangon crangon* were bled directly from the dorsal heart and yielded betwen 150 and 250 μl per animal. For *G. antarcticus,* all bleeding procedures were carried out in the cold room (0 °C ± 1 °C), using equipment and buffers that had been pre-cooled to that temperature.

7.2.3. Preparation of haemocyte lysate supernatants (HLS)

The procedure for HLS preparation followed that described for the shore crab in Chapter 2, page 60, with samples being homogenized in modified *Carcinus* saline (CS II). Haemocyte lysate supernatants for each crustacean were prepared from 25 *C. crangon,* 7 *N. norvegicus,* 6 *G. strigosa* and 3 *G. antarcticus.* Respective volumes of HLS for each species were 2 ml, 5 ml, 4 ml and 1.5 ml. For *G. antarcticuss,* centrifugation and homogenization were carried out at 0 °C. Protein yields in HLS from the different species were standardized to 0.5 mg ml^{-1} for *G. strigosa* and *C. crangon,* 1 mg ml^{-1} for *N. norvegicus* and 0.05 mg ml^{-1} for *G. antarcticus.*

7.2.4. Experiments

For the three temperate water species, *G. strigosa, N. norvegicus* and *C. crangon,* antibacterial activity was investigated using the spread plate method described in Materials and Methods, Chapter 2, page 63. *Psychrobacter immobilis* was used as the test bacterium and experimental and control tubes were incubated at 20 °C for 4 h. The final concentration of bacteria in experimental and control tubes was 2-5 x 10^6. Haemocyte lysate supernatants from each species were serially diluted 1:10 to a dilution of 10^{-3} to ascertain whether activity was present at high titre as shown for

the shore crab, *C. maenas* (see Chapter 4, page 111). Titre of activity is expressed as the reciprocal of the highest dilution at which antibacterial activity is recorded. Experiments were repeated three times.

Galathea strigosa is known to have very low phenoloxidase activity (Smith & Söderhäll, 1991). This permitted antibacterial activity to be monitored spectrophotometrically at 570 nm in microtitre plate assays (as described in Chapter 6, page 154), as well as by the spread plate method. As already demonstrated in Chapters 5 and 6, the microtitre plate assay is only suitable for measuring antibacterial activity in samples that lack phenoloxidase, since gradual activation of the enzyme and the resultant formation of melanin masks underlying events which are taking place. Thus for *G. strigosa,* microtitre plate growth assays were set up in parallel with the spread plate assays, using aliquots of the same samples. Growth was monitored at hourly intervals at 570 nm using a Dynatech microtitre plate reader. Undiluted HLS was used in these experiments, which were repeated three times. Within each experiment, four replicate wells were set up for experimental and control mixtures. Absorbance at 570 nm was plotted against time to produce growth curves.

The protocol for *Glyptonotus* was different. Little is known about host defence in polar invertebrates, so it was decided to use the limited material available to examine the antibacterial activity in *G. antarcticus* haemocytes against both polar and temperate-water bacteria, and additionally to compare antibacterial activities in HLS from animals from vastly different environments. *Glyptonotus antarcticus* comes from a cold but stable environment, whereas *G. strigosa, N. norvegicus* and *C. crangon* reside in warmer and more variable waters.

G. antarcticus HLS was used to challenge *Psychrobacter immobilis, Planococcus citreus* and BS 68 (a marine Antarctic bacterium), and antibacterial activity was determined by the spread plate method. Experiments were carried out at two

incubation temperatures (0 °C and 20 °C) over a period of twelve hours. To allow for the very low protein in *G. antarcticus* HLS (0.05 mg ml^{-1}), a final concentration of 2-5 x 10^5 bacteria ml^{-1} was used in experimental and control mixtures. Aliquots of 450 μl HLS were used in each experiment and samples were not serially diluted. Experiments were repeated three times, apart from that relating to *P. immobilis* which was only repeated twice because after that, all the isopods died.

Marine agar plates were incubated at the appropriate temperatures (see Tables 2.1 & 2.2, pages, 57 & 58) and survival indices (SI) were calculated as given in Materials and Methods, Chapter 2, page 63.

Phenoloxidase activities were recorded for each decapod HLS, using the protocol described in Chapter 2, page 63. Instead of using lipopolysaccharide (LPS), the reaction was elicited with 1 % trypsin (from porcine pancreas. Sigma. Poole, Dorset) for 20 min before addition of the substrate L-dopa (3 mg ml^{-1}). Phenoxidase activity is expressed as units min^{-1} mg $protein^{-1}$, where one unit represents the amount of enzyme activity which gives an increase in absorbance of 0.001 at 490 nm at 20 °C under the experimental conditions.

Limited material was available for *G. antarcticus*, so phenoloxidase activities were not included as part of the investigation into antibacterial activity. However, Smith & Söderhäll (1991), in their survey of phenoloxidase in a range of marine invertebrates, have shown that phenoloxidase activity is negligible in this animal.

7.3. Results

7.3.1. Antibacterial activity

Galathea strigosa, Nephrops norvegicus and Crangon crangon.

Antibacterial activity was found in HLS from all three temperate water species of crustacean. Figures 7.5, 7.6 and 7.7 show the level of activity at each dilution for each species. The activity found in undiluted HLS from all three species compared well with that found in the shore crab, *C. maenas,* with 4 h SI values ranging from 5.05 ± 4.2 for *G. strigosa* to 9.8 ± 3.4 for *N. norvegicus.* In Chapter 2 it has been shown that, typically, the 4 h SI for *P. immobilis* after incubation with *C. maenas* HLS is close to zero. However, it must be remembered, in making such comparisons, that the protein levels in *C. maenas* HLS were higher than those recorded in HLS from *G. strigosa, N. norvegicus* or *C. crangon.* All controls showed growth, although, as might be expected, those bacteria in broth-supplemented medium grew better (SI in excess of 130).

Significant differences ($p < 0.001$) in colony counts were found between experimental tubes and controls for *G. strigosa, N. norvegicus* and *C. crangon* at a titre of 10 (Figures 7.5, 7.6, 7.7). At titres of 100 and 1000, the 4 h SI values for *N. norvegicus* after 4 h were significantly different from buffer controls, with $p < 0.05$ (Figure 7.6); however, at these titres there was a small amount of growth in experimental tubes, suggesting that the antibacterial effect was slight at this dilution (SI values 106.5 ± 9.1 and 104.0 ± 5.7 respectively). For *G. strigosa* there was a significant difference between buffer controls and experimental tubes at a titre of 100 ($p < 0.05$) but not at 1000 (Figure 7.5). Again there was a small amount of growth exhibited at titres of 100 and 1000 (SI 92.6 ± 10.1 and 109.8 ±4.7). For *C. crangon*

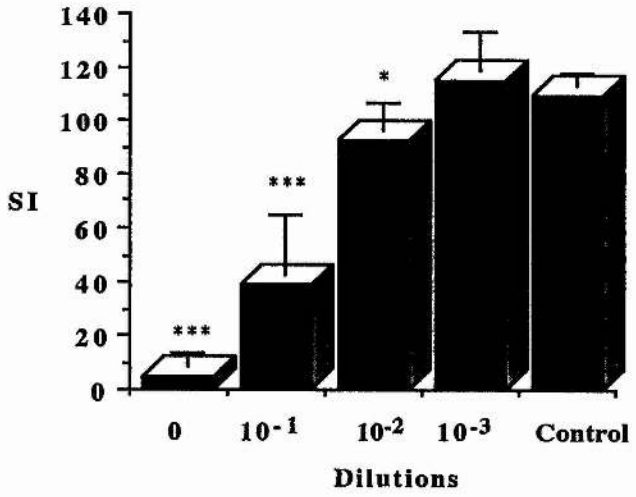
Figure 7.5, 7.6, 7.7

Figure 7.5. Survival of *Psychrobacter immobilis* in haemocyte lysate supernatant (HLS) from *Galathea strigosa*. Samples were diluted 10-fold to 10^{-3}, as described in Materials and Methods, page 183. Only results for controls containing buffer alone are shown. Survival indices (SI) for broth + buffer controls were all in excess of 130. Values are means ± standard deviations (n = 3). Asterisks show levels of significance compared with buffer control at 5 % level (*) and 0.1 % level (***).

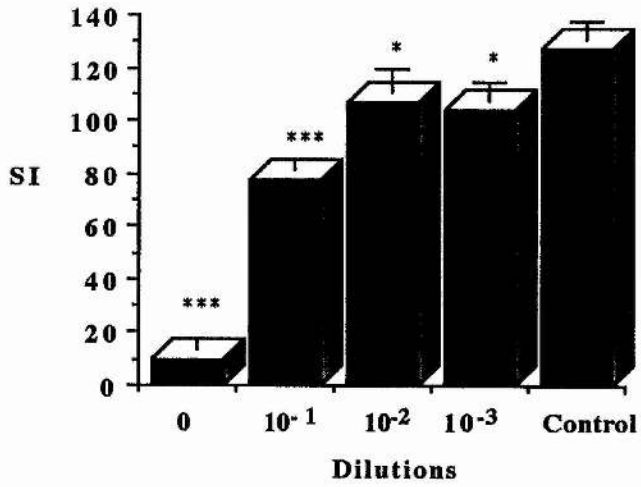
Figure 7.6. Survival of *Psychrobacter immobilis* in haemocyte lysate supernatant (HLS) from *Nephrops norvegicus*. Samples were diluted 10-fold to 10^{-3}, as described in Materials and Methods, page 184. Only results for controls containing buffer alone are shown. Survival indices (SI) for broth + buffer controls were all in excess of 130. Values shown are means ± standard deviations (n = 3). Asterisks show levels of significance compared with buffer control at 5 % level (*) and 0.1 % level (***).

Figure 7.7. Survival of *Psychrobacter immobilis* in haemocyte lysate supernatant (HLS) from *Crangon crangon*. Samples were diluted 10-fold to 10^{-3}, as described in Materials and Methods, page 184. Only results for controls containing buffer alone are shown. Survival indices (SI) for broth + buffer controls were all in excess of 130. Values shown are means ± standard deviations (n = 3). Asterisks show levels of significance compared with buffer control at 5 % level (*) and 0.1 % level (***).

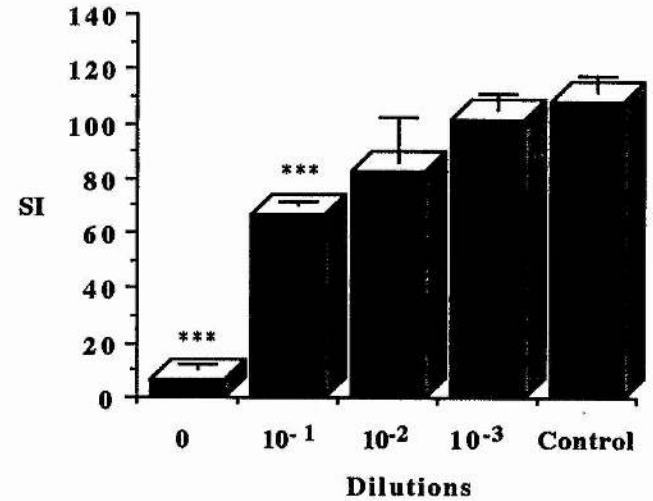
Galathea strigosa



Nephrops norvegicus



Crangon crangon



there were no significant differences between experimental tubes and controls at titres above 10 (Figure 7.7).

Microtitre plate assays for bacteria incubated in Galathea HLS

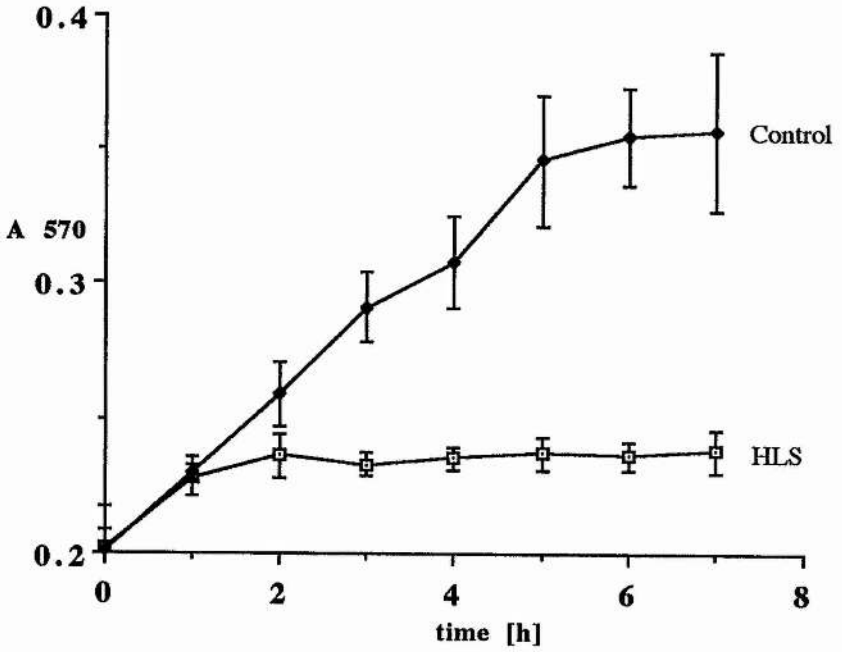
Growth was monitored over a period of 6 - 7 h at 570 nm and during that time the controls, containing *G. strigosa* HLS and buffer only, showed only a small change in overall absorbance (0.002 at 570 nm). Figure 7.8 shows a typical growth curve obtained after 7 h incubation of *P. immobilis* with HLS and with buffer. In experimental wells there is a small initial increase in turbidity in the first hour of incubation but after that the absorbance measurements remain constant for the remaining six hours. For controls there is a steady increase in absorbance which starts to plateau at 5 h (Figure 7.8). Taking these results in conjunction with the spread plate assays, it appears that antibacterial activity is present in *G. strigosa* HLS but is not due to lysis.

Glyptonotus antarcticus

Glyptonotus antarcticus HLS showed activity against the bacteria BS 68, *P. citreus* and *P. immobilis in vitro* (Figures 7.9, 7.10, & 7.11). A maximum reduction in the survival index (SI) for all bacteria was observed by 4 h incubation. The degree of activity differed according to the test bacteria and the incubation temperature, but in general antibacterial activity was stronger at 20 °C than at 0 °C, particularly in the first 4 h of the experiment. Nevertheless, *G. antarcticus* HLS was still able to reduce colony counts of BS 68, *P. citreus* and *P. immobilis* within 4 h at 0 °C. Regrowth of *P. immobilis* occurred after 4 h at 20 °C and in BS 68 after 2 h at 0 °C. At no time did SI values drop to the low levels found in *C. maenas, G. strigosa, C. crangon* and *N. norvegicus*. Again, it should be remembered that the protein levels in *G. antarcticus* HLS were extremely low, although the level of bacterial challenge had been reduced by a factor of ten to compensate for this. None of the bacteria tested showed any

Figure 7.8

Figure 7.8. Microtitre plate assay to show the effect of haemocyte lysate supernatant (HLS) from *Galathea strigosa* on *Psychrobacter immobilis*. These experiments were designed to determine whether lytic activity was present in *G. strigosa* HLS. Experiments were carried out in microtitre plates and growth was monitored at 570 nm as described in Materials and Methods, page 184. Controls contained modified *Carcinus* saline (CS II) as a substitute for HLS. Values are means ± standard deviations (n = 3).


0.4
A 570
0.3
0.2
0
2
4
6
8
time [h]
Control
HLS

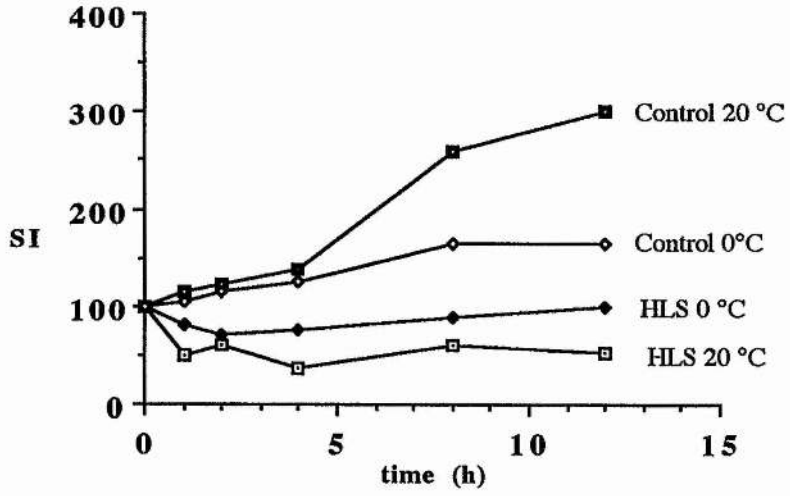
Figures 7.9, 7.10 & 7.11

Figure 7.9. Survival of BS 68 after incubation in *G. antarcticus* haemocyte lysate supernatant (HLS) at 0 °C and 20 °C. HLS was prepared as described in Materials and Methods, Chapter 2, page 60. Survival indices (SI) relate to a typical experiment, and were calculated as given in Chapter 2, page 63.

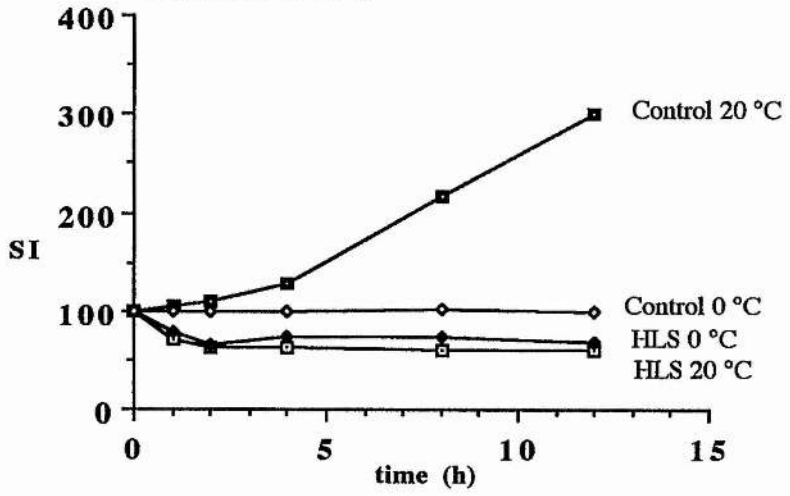
Figure 7.10. Survival of *Planococcus citreus* after incubation in *G. antarcticus* haemocyte lysate supernatant (HLS) at 0 °C and 20 °C. HLS was prepared as described in Materials and Methods, Chapter 2, page 60. Survival indices (SI) relate to a typical experiment, and were calculated as given in Chapter 2, page 63.

Figure 7.11. Survival of *Psychrobacter immobilis* after incubation in *G. antarcticus* haemocyte lysate supernatant (HLS) at 0 °C and 20 °C. HLS was prepared as described in Materials and Methods, Chapter 2, page 60. Survival indices (SI) relate to a typical experiment, and were calculated as given in Chapter 2, page 63.

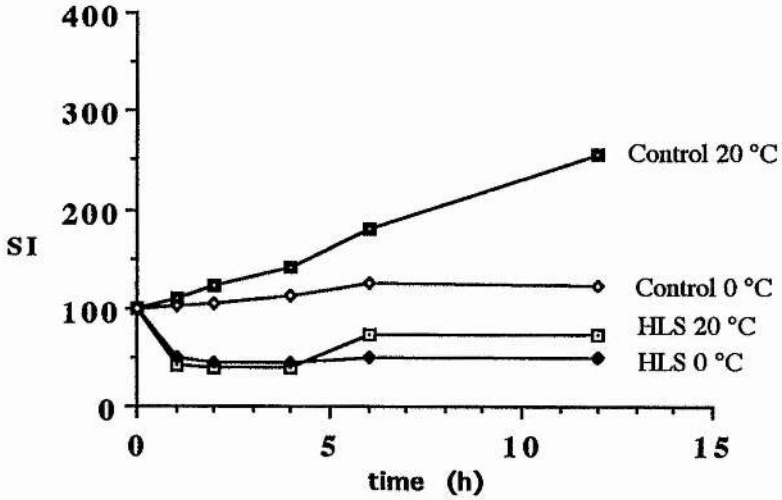
BS 68. Marine Antarctic bacterium



Planococcus citreus



Psychrobacter immobilis



regrowth after 12 h in isopod HLS at 20 °C, although BS 68, but not *P. citreus,* regrew after 4 h incubation at 0 °C.

7.3.2 Phenoloxidase activities

Phenoloxidase activities for *G. strigosa, N. norvegicus* and *C. crangon* are shown in Table 7.1. It can be seen that activities are high for *N. norvegicus* and *C. crangon* (>1000 units min^{-1} mg $protein^{-1}$ in both instances) and low by comparison in *G. antarcticus* (16 ± 1.4 units). In all cases there is enhanced activity over controls.

7.4. Discussion

Antibacterial activity was found in undiluted HLS from the four crustaceans tested. For *G. strigosa, N. norvegicus* and *C. crangon,* there was also measurable activity at a titre of ten. At higher dilutions bacterial growth was generally present in experimental mixtures, albeit not to the same degree observed in controls.

It has aready been shown in Chapter 4 that the antibacterial effect of*C. maenas* HLS is not due to phenoloxidase itself, and it would seem, from the results presented here, that the same is likely to be true for *G. strigosa, N. norvegicus, C. crangon* and *G. antarcticus.* Certainly, the antibacterial activity present in the three decapods is powerful in undiluted HLS (SI *circa* 10 for all after 4 h) and yet phenoloxidase activity is relatively weak in one of these, namely *Galathea.* By comparison, bacteria were less susceptible to *Glyptonotus* HLS, (which also lacks phenoloxidase activity), but nevertheless antibacterial activity was present, even at 0 °C, and where regrowth of bacteria occurred it was generally after the elapse of at least four hours.

The differing protein levels in HLS from the four species of crustacea make it hard to draw comparisons in terms of the effectiveness of the *in vitro* antibacterial

Table 7.1. Phenoloxidase activity in HLS from *Galathea strigosa, Nephrops norvegicus* and *Crangon crangon*

Species	Phenoloxidase activity[1] (units min^{-1} mg $protein^{-1}$)	
	HLS[2] Trypsin activated[3]	Control[4]
Galathea strigosa	16 ±2	10 ± 2
Nephrops norvegicus	1500 ± 285	322 ± 64
Crangon crangon	2460 ± 158	150 ± 28

[1]Phenoloxidase activity measured at 490 nm as described in Materials and Methods,Chapter 2, page 63. One unit is defined as the amount of enzyme activity that gives an increase in absorbance of 0.001 at 490 nm under the experimental conditions.

[2]HLS: Haemocyte lysate supernatants from each crustacean species, prepared as described in Materials and Methods, page 183.

[3]Trypsin from bovine pancreas used at a concentration of 0.1 % in modified *Carcinus* saline (CS II) and preincubated with HLS before addition of L-dopa as the substrate.

[4]Control: Controls contained CS II as a substitute for trypsin.

Values are means ± standard deviations (n = 3).

activity in the haemocytes of the various crustaceans. In general terms there was little difference in the activities displayed by *G. strigosa, N. norvegicus* and *C. crangon,* in spite of the fact that *N. norvegicus* HLS contained twice as much protein as that from *G. strigosa* or *C. crangon.*

In the experiments with *C. maenas* HLS, where protein levels were adjusted to 1.8 mg ml-1 (see Chapter 4, page 101) activity was present at a titre of at least 1,000 (equivalent to a protein concentration of approximately 2 μg ml^{-1}), whereas in *G. strigosa, N. norvegicus* and *C. crangon,* significant reductions in colony counts over time zero were only found at a maximum titre of 10, which is equivalent to protein concentrations of 50, 50, and 100 μg protein ml^{-1} respectively. It was noted, however, that at higher dilutions *P. immobilis* grew more slowly in HLS from these animals than in it did in the controls, even in the nutrient-depleted buffer, so there was still some growth inhibition present in diluted HLS. It would be inappropriate to speculate on the *in vivo* implications of these observations, since at present we have no quantitative data with respect to antibacterial factors in haemolymph, and to what extent antibacterial factors become diluted after exocytosis from granular cells is not known and, indeed, may be impossible to define.

Glyptonotus is an interesting animal to work on for a number of reasons, quite apart from its lack of phenoloxidase activity in the haemocytes. Our understanding of invertebrate host defence mechanisms is largely based on observations of temperate species and very little is known about the defence capabilities of species from extreme environments. This study provides an initial investigation into one aspect of immunocompetence in a polar invertebrate and has shown that *G. antarcticus* can mount an efficient defence response against some Gram-positive and Gram-negative bacteria despite living in extreme cold - the normal temperature range for these animals is narrow (-2 °C to +2 °C) compared with that for animals such as *C. maenas,* which occupies the equivalent ecological niche in temperate waters. Only three species of

bacteria were tested because of the limited number of animals available, and it is always possible that the choice of organisms was fortuitous. It would be useful to extend studies on this animal to include phagocytosis and agglutination as well as more detailed investigation of the antibacterial factors in the haemocytes. In addition, since the proPO system is implicated in invertebrate recognition, it would be of interest to determine whether the proPO system is lacking in ite entirety in these isopods or whether, as with the horseshoe crab, *Limulus polyphemus,* it is just the terminal component (phenoloxidase) that is missing. In this context it was interesting to note that, following bleeding of these animals, no evidence of melanization developed at the site of injury, whereas in *C. maenas,* for example, heavily melanized areas always developed at the point of needle penetration.

Global warming has become a matter of concern in recent years and it is likely that marine communities in high latitudes will be the most vulnerable to small climatic shifts. Members of these communities are highly adapted to their environment and many aspects of their biology, including immunocompetence, may be affected by thermal change. Further studies on polar invertebrates are needed if we are to understand and assess the impact of climatic change in these fragile communities.

The general conclusion to be drawn from the results in this chapter is that the phenomenon of antibacterial activity in crustacean haemocytes is unlikely to be restricted to just one or two species and probably occurs throughout the Class. The question of whether activity is connected to activation of the prophenoloxidase cascade is still not resolved (and is likely to remain that way until the proPO pathway is fully understood), although evidence is accumulating which suggests that antibacterial activity in the haemocytes is a separate entity. The presence of antibacterial factors in animals such as *Gl. antarcticus* and *G. strigosa,* which show low or negligible phenoloxidase activity, goes some way to validate this point of view.

Chapter 8
General Discussion

General Discussion

This thesis has presented evidence that *in vitro* antibacterial activity in the haemocytes of the shore crab is active against a range of bacteria, both Gram-positive and Gram-negative, and can be attributed to at least three factors with different molecular weights. It would appear that these factors reside in the granular cells, since activity is present in the latter but absent from hyaline cells and plasma. Antibacterial activity in *Carcinus maenas* has no requirement for divalent cations, is active at high titre and is heat stable. The relationship between antibacterial activity and the prophenoloxidase activating (proPO) system remains unclear, and aspects of the central hypothesis (that antibacterial activity is linked in some way to the proPO system) have still to be explored. Nevertheless, it appears that phenoloxidase and an activating serine protease are not directly responsible for antimicrobial effects. Whether other factors in the cascade are involved is a matter for further research.

Low levels of bacterial agglutination have been recorded for haemocyte lysate supernatants (HLS) and low levels of lysozyme-like activity are also present, but to what extent these contribute to the overall antimicrobial effect has not been determined. In general, invertebrate agglutinins have high molecular weights, usually in excess of 150 kDa (see Table 1.2, page 31), whereas the antibacterial activity in *C. maenas* HLS was associated with proteins with molecular weights 4 kDa, 34 kDa and 72 kDa. Since these molecular weights are only approximations, the possibility that agglutination resides in one of these fractions (possibly the 72 kDa fraction) should be considered. As regards the presence of lysozyme, it has been shown in Chapter 6 that none of the antibacterial activity could be attributed to a protein with a molecular weight close to that of lysozyme (14 - 15 kDa).

Although antibacterial activity in *C. maenas* HLS is heat stable, a gradual reduction in the effectiveness of the reaction is observed with increasing temperature.

This could be explained by partial/sequential inactivation of the component factors, whereby each have a different thermal tolerance. For example, the active fraction with an approximate MW of 74,000 kDa is probably more easily heat-denatured than the molecule with an estimated MW of 4,000 KDa, since the larger molecule is likely to contain more disulphide bonds in the native state (Stryer, 1981).

Antibacterial factors are ubiquitous in vertebrate and invertebrate body fluids and are not confined to the blood. The fluid from the purple gland of the sea hare, *Aplysia kurodai,* for example, has strong microbicidal properties (Yamazaki *et al.,* 1990), as does the superficial mucus of terrestrial molluscs such as *Achatina fulica* Férussac (Iguchi *et al.,* 1982), and in humans it has been demonstrated that amniotic fluid has similar properties (Schlievert *et al.,* 1976; Feingold *et al.,* 1979). In the crustacean, *Homarus americanus,* considerable antibacterial activity resides in the hepatopancreas, and in insects inducible immune proteins are apparently synthesised by the fat body (Faye & Wyatt, 1980).

The mode of action of the different antibacterial factors in *C. maenas* HLS needs to be fully elucidated. There appears to be a combination of bacteriostatic and bacteriolytic effects which may operate individually or jointly to eliminate bacteria. As already enumerated in the discussion in Chapter 6, there are a variety of ways in which bacterial killing or growth inhibition can be brought about. Resolution of this question for *C. maenas* will require the harnessing of molecular biology techniques to examine the structure of the antimicrobial factors and their effects on bacterial cell walls.

In human polymorphonuclear leucocytes, the azurophil, a specialized lysosome of neutrophils, contains two families of antibacterial proteins (see review by Gabay and Almeida, 1993), one of which, the defensins, were originally discovered by Lehrer *et al.* (see review 1991) and occur in invertebrates (Lambert *et al.,* 1989; Miyata *et al.,*

1989), as well as mammals. Defensins are small cationic peptides; they comprise 29-43 amino acids with three disulphide bonds, appear to act by cell membrane disruption and have a broad spectrum effect (Gabay & Almeida, 1993). Structurally they exhibit differences when compared to other antimicrobial peptides, such as the cecropins of insects and the magainins of amphibians (Zasloff, 1987), which all have an α-helical structure and form channels in artificial membranes (see review by Vaara, 1992). Defensins, by contrast, are ß-sheets, which suggests that they may increase cell membrane permeability in a different way (Gabay & Almeida, 1993). One interesting feature about some of these antimicrobial peptides (e.g. defensin and magainin) is that they bind to LPS; it was found in Chapter 5 that the antibacterial activity in *C. maenas* HLS could be adsorbed away by preincubation of HLS with LPS, so there may a cationic peptide component to the antibacterial activity in crab haemocytes.

Not all antimicrobial molecules are small. Larger cationic peptides occur in the eosinophils and neutrophils of mammals (Vaara, 1992) and in the haemocytes of invertebrates (Boman & Hultmark, 1987). For example, lactoferrin, a 78 kDa glycoprotein is found in eosinophils (Ellison, *et al.*, 1988) and a bactericidal /permeability increasing protein (BPI, MW 58 kDa) is found in neutrophils (Elsbach & Weiss, 1993). In invertebrates, insect attacins reduce the synthesis of major outer membrane proteins (Engström *et al.*, 1984; Carsson *et al.*, 1991) and potentiate the antibacterial activity of the smaller cecropins. There is relatively little information available with respect to this aspect of crustacean host defence, but it seems, from the work described in this thesis, that antibacterial activity in *C. maenas* haemocytes is multifactorial. It is not impossible therefore, that the molecules responsible bear similarities to one or more of those described above for insects or vertebrates.

The incubation times used for the experiments in this thesis were short (usually 4 - 6 h) since the intention was to examine early antibacterial effects, rather than the later

ones. Smith & Ratcliffe (1980a) have shown that, in *C. maenas,* injected bacteria are rapidly cleared from the circulation (75 % clearance in 5 min; > 90 % clearance over 6 h) to the gills and other sites where they are sequestered in haemocyte clumps. Given this rapidly effective system, one could argue that there is an advantage in having an antimicrobial response that is swift and augments phagocytosis - Boman (1991) has set out a cogent argument for a response that is mounted rapidly and effectively, such as occurs with the inducible antibacterial proteins in insects (see Chapter 1, page 51). For some bacteria, such as *Pseudomonas* 1-1-1 and *Psychrobacter immobilis* (see Chapter 2, page 65), antibacterial activity in *C. maenas* HLS resulted in survival indices of < 10 within 1h, which indicates a response that is both rapid and profound. In addition, it has been shown by White *et al.* (1985) that antibacterial activity is present in the haemocyte clumps in the gills of *C. maenas.* This may well arise as a consequence of localized release of antibacterial factors from granular cells, during the process of bacterial sequestration.

The question of inducibility has not been addresssed in this thesis. In insects there is plentiful evidence that antibacterial proteins are inducible (see review by Boman, 1986), whilst early work by Evans and co-workers (see Evans *et al.* 1968, 1969a,b) has provided some evidence that this may also be true for crustaceans. However, as already discussed in Chapter 1, the crustacean response has not been properly explained and there are no supporting studies relating to protein synthesis in tissues, as there are for insects. One problem here is that baseline measurements of antibacterial activity are difficult to establish in populations of animals that have been collected from the wild. Whilst it is possible to rear many laboratory animals under gnotobiotic conditions, it is unlikely that this will ever be the case for crustaceans. It has been shown here that antibacterial activity in HLS from *C. maenas* is generally very strong, but we have no way of knowing whether this represents normal baseline activity or is a consequence of prior bacterial challenge. Colwell *et al.* (1975) have

recorded that for *Callinectes sapidus* (the blue crab), the haemolymph of apparently healthy animals is not sterile, so it may be that the crab immune system is regularly engaged in dealing with potential pathogens. Where inducibility has been studied in crustaceans, the response times for maximum effect have ranged from 2-7 days (see review by Smith & Chisholm, 1992), which is unlikely to confer any immediate protection on the host. Nevertheless, a role can be envisaged for molecules that persist in the haemolymph acting as long term disinfectants and protecting the host against recurrent microbial challenge.

In *C. maenas*, the antimicrobial factors reside in the granular cells, which also contain the prophenoloxidase (proPO) activating system, and will only be released when exocytosis of these cells is triggered by suitable non-self molecules such as ß, 1-3 glucans (Smith & Söderhäll, 1983a). In this way, wasteful or harmful release of antibacterial factors may be avoided. This constitutes an evolutionarily sound strategy and would ensure that antibacterial factors are utilized with maximum effectiveness and economy. As mentioned previously in Chapter 1, crabs are particularly vulnerable to injury and infection during ecdysis, and at this time large numbers of haemocytes are redeployed to epidermal areas where the granular cells are engaged in the important task of tanning the new exoskelton (Vacca & Fingermann, 1983). It may be valuable that the cells engaged in this process also contain antibacterial factors which could provide additional immune protection at the very site which is most vulnerable.

It seems that the proPO system, which may have evolved from a clotting pathway into a protective tanning mechanism, has conferred other advantages on the host animals with respect to recognition of non-self molecules. Yet immunocompetence in crustaceans cannot rest entirely on phenoloxidase activity, since *G. antarcticus* and *G. strigosa* can mount an effective antibacterial response when phenoloxidase is either substantially reduced or lacking. This is true also of the horseshoe crabs in which the

terminal component of the cascade, phenoloxidase, is missing (Söderhäll & Smith, 1986a).

The understanding of invertebrate immune systems is not merely an academic or intellectual exercise. Invertebrates have made significant contributions to medicine and there are several interesting reviews which highlight potentially valuable therapeutic substances which have been identified in a whole range of invertebrates (see for examples, Nigrelli *et al.*, 1967; Pettit *et al.*, 1981; Ravindranath & Cooper, 1984; Smith, 1991). The contribution of *Limulus polyphemus* to the development of an endotoxin assay has already been mentioned in Chapter 1. The assay is based on the ability of amoebocyte extracts to undergo gelation in the presence of minute amounts of endotoxin (Levin & Bang, 1964) and has led to the development of diagnostic kits which are widely used to detect endotoxin in intravenous fluids, water and vaccines, amongst others. *Limulus* amoebocyte lysate is also sensitive to pyrogenic exotoxins from group A Streptococci (Brunson & Watson, 1976) although the exotoxin reaction is many times less sensitive than that produced by the Gram-negative endotoxin (Brunson & Watson, 1976). According to Thomas (1976), horseshoe crabs are popular but expensive laboratory animals and explorations are under way to find other marine invertebrates with similar coagulable proteins.

Drug therapy for human ailments has its origins in natural products and there is no reason to suppose that the source of useful substances has been exhausted. Apart from the antimicrobial molecules already discussed, there are numerous potentially useful invertebrate cytotoxic and antineoplastic compounds which have been isolated from invertebrates as diverse as tunicates (Rinehart *et al.*, 1983) and sea hares (Pettit *et al.*, 1987; Kamiya *et al.*, 1986). In addition, lectins from a variety of invertebrate sources, including *Helix pomatia* (edible snail), *L. polyphemus* (horseshoe crab), and *Limax flavus* (slug) have become useful tools in medical research (Damjanov, 1987). They are routinely applied to the study of cell surface determinants, and are widely

maturation markers in the monitoring of functional and developmental changes, and also in the identification of species, strain and group differences (see for example, Virtanen *et al.,* 1986; and review by Damjanov, 1987). Lectins are also used as probes in pathology - for example, in the study of tumour histogenesis and in the study of the relationship of tumours to inflammatory and pre-neoplastic conditions (Miettinen, 1983).

Invertebrates have occasionally provided models for human disease, an example of which may be found in the studies of the haemic proliferative disorder in bivalve molluscs (Farley, 1969) This condition has several features in common with vertebrate neoplasia and consequently has generated much interest in the field of medical research (Reinisch *et al.,* 1983). It has also been noted that there is an increase in prevalence of the disease in polluted areas (Reinisch *et al.,* 1984), which has led to some speculation as to the role of pollutants in the aetiology of neoplasia.

There is presently much concern about the immunosuppressive influence of certain pollutants on marine or freshwater invertebrates and interest is being generated in the development of novel bio-markers for subacute toxic effects. In molluscs, exposure of the clam, *Mercenaria mercenaria,* to heavy metals has been shown to cause destabilisation of the haemocyte lysosomal membrane with the consequent release of acid phosphatase into the haemolymph (Suresh & Mohandas, 1990). Continued exposure to pollutants inhibits its activity and blocks further synthesis of the enzyme by the blood cells (Suresh & Mohandas, 1990). The effect of environmental factors, such as pollutants, on humoral immunity in crustaceans is very poorly understood, despite the almost universal use of crustacean species in LC_{50} toxicity tests. Recently, Smith & Johnston (1991) have found that short term exposure of the shrimp, *Crangon crangon,* to sub-acute concentrations of polychlorinated biphenols (PCBs) *in vitro* results in depression of phenoloxidase activity in haemocytes and suppression of the total blood cell count. This study also

revealed that there is a differential haemal response according to the PCB congener used, and in a manner that could not be predicted from immunological studies in vertebrates (Johnston *et al.*, 1991; Smith & Johnston, 1991). However, the potential of the non-cellular defences as indicators of environmental perturbation is still unexplored despite the obvious advantages that blood parameters offer in terms of non-sacrificial accessibility, sensitivity and known physiological importance.

With respect to disease control in cultivated populations of crustaceans (see Chapter 1 for an account of disease problems in crustacean aquaculture), the absence of clonally derived specific antibody-type molecules in the host defence repertoire means that the production of vertebrate-style vaccines is unrealistic. Whether it will be possible to stimulate the synthesis of other broad spectrum antimicrobial agents for prolonged periods is still unproven. To date there is only sparse evidence that immunization confers protection on the host through the induction of microbicidins. Enhanced secondary and tertiary responses have been noted in only two species of spiny lobster (Evans *et al.*, 1969b; Weinheimer *et al.*, 1969a) and in neither case was the effect large. The injection of non-self materials adversely affects the number of circulating haemocytes in crustaceans (Smith & Ratcliffe, 1980a,b), which could abrogate any apparent beneficial effects that immunization might have. In order to promote immunocompetence in crustaceans, further research into the synthesis and mobilisation of of antimicrobial factors, whatever their source, is necessary.

The phylogenetic implications of research into invertebrate immune systems should not be ignored. As pointed out by Smith (1991), an understanding of the origins of immune mechanisms may help in our understanding of allergies, tumours and healing responses. Already studies of colonial animals, particularly tunicates (which occupy a phylogenetically strategic position in the Animal Kingdom), have revealed histocompatibility systems that are markedly similar to the vertebrate MHC (see Cooper *et al.*, 1992, for review). Certain features of invertbrate immune systems

seem to have been conserved during evolution, particularly with regard to non specific responses. For example, cytotoxicity occurs in both invertebrates and vertebrates, although there is a more complex array of cytotoxic cells in vertebrates (Cooper *et al.*, 1980). Furthermore, the clotting system of horseshoe crabs is a cascade with features in common with the clotting cascade that arises from human platelets (Levin, 1985). Enzyme cascades are an effective way of amplifying and regulating responses so it should not be a surprise to find that the general features of such a system have been conserved throughout evolution. It would be interesting and informative to extend the studies of antibacterial factors in crab haemolymph to see to what extent they resemble antibacterial molecules in other animals.

It is clear that invertebrates are a rich source of novel compounds and also engage in sometimes unique strategies with regard to host defence. As far as crustaceans are concerned, and with regard to the main experimental animal used for the work in this thesis, there is still much to be resolved. Not only are there large voids in our understanding with respect to recognition, but also there are still many unanswered questions with regard to the antibacterial factors in the haemocytes. For the future there is room for further research to establish the exact number and molecular weights of antibacterial factors as well the application molecular biology tools to ascertain the mechanism of action for each factor.

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Appendices

Appendix I. Formulation of buffers

Complete *Carcinus* saline (CS I)[1] pH 7.4

0.58 M NaCl

7.0 mM KCl

13 mM $CaCl_2$ $6H_20$

26 mM MgCl2 6H20

0.6 mM Na_2HPO_4. 12 H_20

50 mM Tris (hydroxy methyl) methylamine

42.5 ml 1 M HCl

Distilled water to 1 litre

Modified *Carcinus* saline (CS II) pH 7.4

0.58 M NaCl

7.0 mM KCl

20 mM $CaCl_2$ $6H_20$

0.6 mM Na_2HPO_4. 12 H_20

50 mM Tris (hydroxy methyl) methylamine

42.5 ml 1 M HCl

Distilled water to 1 litre

Citrated Cacodylate Buffer (CAC) pH 7.0

45 mM NaCl

100 mM Trisodium citrate

10 mM Sodium cacodylate

Marine anticoagulant (MAC) pH 4.6

0.450 mM NaCl

100 mM Glucose

30 mM Trisodium citrate

26 mM Citric acid

10 mM EDTA

Tris buffer pH 8.0

200 mM Tris hydroxymethyl methylamine (125 ml)

100 mM HCl (137.5 ml)

Dilute to 500 ml with distilled water

0.06 M phosphate buffer pH 6.5 (Salt supplemented)

65 mM Na_2 HPO4. $2H_2O$ (30 ml)

65 mM KH_2PO_4 (70 ml)

0.58 g NaCl

[1] After Smith & Ratcliffe (1978)

Appendix IIa

Statistical data from Chapter 3

Appendix IIa. *Carcinus maenas* raw data for seasonal experiments[1]

Month[2]	HLS Protein[3] (mg ml^{-1})	4 h SI[4]	Water temperature (°C)	Haemocyte count per crab (x 10^7)
January	1.0	0.001	8.0	1.49 0.63 3.57 2.70 1.38
	1.0	0.100	7.0	2.37 1.83 1.93 0.66 1.02
	1.1	0.010	7.0	1.48 2.25 1.91 1.52 1.88
February	0.6	35.800	3.5	2.07 2.75 2.60 2.25 0.92
	0.6	25.000	3.0	2.93 3.24 3.31 1.65 3.08
	0.7	30.900	3.5	2.18 2.03 0.44 0.48 0.46
March	1.0	2.100	7.0	2.16 2.44 2.01 1.21 2.54
	1.3	10.000	7.5	2.05 3.47 2.45 2.48 2.90
	1.6	3.6000	7.5	1.59 3.90 2.05 2.24 2.14
April	1.3	2.700	9.0	3.38 2.12 3.22 3.20 2.49
	1.8	0.800	9.0	2.83 3.27 4.33 4.66 3.15
	1.3	2.000	10.0	5.82 3.04 3.23 2.53 5.85
May	1.9	1.200	13.0	3.32 4.29 5.99 7.18 7.76
	1.6	0.200	12.0	2.50 3.71 2.05 1.78 1.07
	1.6	0.500	12.0	3.41 4.20 2.75 1.99 2.02
June	1.7	0.001	14.0	3.17 1.72 6.01 2.97 3.24
	1.8	0.002	14.0	2.67 3.67 3.57 3.65 2.68
	2.0	0.001	14.0	3.93 2.99 3.46 3.86 2.55

July	1.2	0.001	15.0	1.97 3.18 3.57 2.31 3.94
	1.6	0.003	15.0	2.84 3.76 3.99 2.78 4.10
	1.8	0.005	15.5	3.89 2.44 2.55 3.44 3.65
August	0.8	16.800	16.5	0.73 0.89 0.94 1.05 1.06
	0.7	20.500	16.5	3.82 2.84 0.96 0.39 2.87
	0.5	15.600	16.0	1.63 0.74 0.67 0.87 1.68
September	1.3	0.003	13.0	2.73 3.66 3.60 5.12 2.65
	1.2	0.004	13.0	1.66 1.29 3.35 4.16 3.63
	1.3	0.001	12.0	2.05 1.35 2.11 1.04 1.97
October	1.1	0.200	10.0	1.46 2.53 3.66 4.19 1.52
	1.3	0.300	10.0	2.43 3.20 2.67 1.99 1.78
	1.2	0.200	10.0	2.18 1.89 2.22 3.10 2.03
November	0.9	0.300	8.0	2.56 0.93 2.21 1.77 1.34
	1.1	0.100	8.0	2.75 3.38 3.15 3.07 4.22
	1.2	0.200	8.5	2.89 2.67 2.54 3.04 3.07
December	1.2	0.001	7.5	4.50 4.20 2.81 2.08 1.41
	1.1	0.001	7.5	3.12 2.34 1.94 2.08 1.87
	1.1	0.021	7.0	1.61 1.54 4.03 2.78 2.17

[1]Experiments carried out as described in Materials and Methods, Chapter 3, page 79, using *Psychrobacter immobilis* as test organism.

[2]Experiments performed on three consecutive days of each month.

[3]HLS: haemocyte lysate supernatant. Protein measured as described in Materials and Methods, Chapter 2, page 64.

[4]SI: survival index calculated as shown in Chapter 2, page 63. Control SI values are not shown, but all were in excess of 100 and showed bacterial growth.

Appendix IIb. Statistical data from Chapter 3.Mean monthly data from seasonal observations.
(January 1991-February 1992)

Month	SI[1] Experimental	SI[1] Control	Water temp (°C)[2]	Protein (mg ml^{-1})[3]	Haemocytes (x 10^7 ml^{-1})[4]
January	0.037 ± 0.055	128.7 ± 14.8	7.3 ± 0.6	1.0 ± 0.1	1.775 ± 0.767
February	30.567 ± 5.408	118.7 ± 16.7	3.3 ± 0.3	0.7 ± 0.1	2.026 ± 1.025
March	5.233 ± 4.196	131.6 ± 9.9	7.3 ± 0.3	1.3 ± 0.3	2.375 ± 0.670
April	1.840 ± 0.975	115.8 ± 7.7	9.3 ± 0.6	1.4 ± 0.4	3.541 ± 1.130
May	0.633 ± 0.513	125.5 ± 5.4	12.3 ± 0.6	1.6 ± 0.3	3.601 ±1.999
June	0.001 ± 0.001	138 2 ± 6.4	14.0 ± 0.0	1.9 ± 0.1	3.343 ± 0.943
July	0.003 ± 0.002	129.7 ± 8.3	15.2 ± 0.3	1.6 ± 0.3	3.273 ± 0.695
August	17.700 ± 2.476	119.4 ± 3.5	16.3 ± 0.3	0.6 ± 0.1	1.409 ± 0.994
September	0.003 ± 0.002	181.5 ± 63.1	12.7 ± 0.6	1.2 ± 0.1	2.665 ± 1.199
October	0.233 ± 0.058	141.2 ± 21.0	10.0 ± 0.0	1.2 ± 0.1	2.457 ± 0.786
November	0.200 ± 0.100	115.3 ± 10.4	8.2 ± 0.3	1.1 ± 0.1	2.639 ± 0.823
December	0.008 ± 0.012	108.2 ± 9.5	7.3 ± 0.3	1.1 ± 0.1	2.567 ± 0.992

[1]SI: Survival index calculated as given in Chapter 2, page 63. Values are means with standard deviations (n=3). Incubation time, 4 h.

[2]Mean seawater temperature for three consecutive days of each month.

[3]Protein measured as described in Materials and Methods, Chapter 2, page 64.

[4]Mean number of haemocytes ml^{-1} whole haemolymph calculated for fifteen animals each month.

Appendix IIc. Statistical data from Chapter 3. Probability values and t-statistics for Student's t-test in relation to monthly HLS protein levels.

Comparison[1]		t-statistic[2]	p value[3]	Significance[4]
January	- February	8.2593	0.001	***
	March	-1.5665	0.192	ns
	April	-1.7111	0.162	ns
	May	-6.3120	0.003	**
	June	-8.5000	0.001	***
	July	2.7559	0.051	ns
	August	4.8270	0.008	**
	September	-5.0131	0.007	**
	October	-2.4495	0.070	ns
	November	-3.9630	0.719	ns
	December	-2.0642	0.108	ns
February	- January	-8.2593	0.001	***
	March	-3.7774	0.019	*
	April	-3.5030	0.025	*
	May	-9.8298	0.001	***
	June	-12.3919	0.000	***
	July	-4.8550	0.008	**
	August	0.4121	0.701	ns
	September	-12.6747	0.000	***
	October	-8.0299	0.001	***
	November	-4.6628	0.010	**
	December	-9.8845	0.001	***
March	- January	1.5665	0.192	ns
	February	3.7774	0.019	*
	April	-0.3493	0.744	ns
	May	-2.0166	0.114	ns
	June	-2.7827	0.050	*
	July	-0.9319	0.404	ns
	August	3.6698	0.021	*
	September	0.2343	0.826	ns
	October	0.6022	0.580	ns
	November	1.2507	0.279	ns
	December	1.0151	0.367	ns

[1]Months are February 1991-January 1992.
[2]t- statistic for 4 degrees of freedom as given by Statworks statistics programme.
[3]The null hypothesis states that there is no difference between the mean values of the samples. Differences are significant at the 5% level if $p < 0.05$; 1% level if $p < 0.01$; 0.1% level if $p < 0.001$.
[4]Asterisks indicate levels of significance: * = 0.05 % ** = 0 01 % *** = 0.001 %

	Month	t-statistic	p value	Significance
April -	January	1.7111	0.162	ns
	February	3.5030	0.025	*
	March	0.3493	0.744	ns
	May	-1.12990	0.264	ns
	June	-1.9167	0.128	ns
	July	-0.4887	0.651	ns
	August	3.4731	0.026	*
	September	0.6325	0.561	ns
	October	0.9258	0.407	ns
	November	1.4595	0.218	ns
	December	1.2649	0.275	ns
May -	January	6.3120	0.003	**
	February	9.8298	0.001	***
	March	2.0166	0.114	ns
	April	1.12990	0.264	ns
	June	-1.0000	0.374	ns
	July	0.8220	0.457	ns
	August	8.3795	0.001	***
	September	4.1110	0.015	*
	October	4.3301	0.012	*
	November	4.8525	0.008	**
	December	5.3759	0.006	**
June -	January	8.5000	0.001	***
	February	12.3919	0.000	***
	March	2.7827	0.050	*
	April	1.9167	0.128	ns
	May	1.0000	0.374	ns
	July	1.5213	0.203	ns
	August	10.1272	0.001	***
	September	6.0104	0.004	**
	October	6.0083	0.004	**
	November	10.1272	0.001	***
	December	7.4246	0.002	**

	Month	t-statistic	p value	Significance
July -	January	-2.7559	0.051	ns
	February	4.8550	0.008	**
	March	0.9319	0.404	ns
	April	0.4887	0.651	ns
	May	-0.8220	0.457	ns
	June	-1.5213	0.203	ns
	August	4.6863	0.009	**
	September	1.4856	0.212	ns
	October	1.7961	0.147	ns
	November	2.3652	0.077	ns
	December	2.2283	0.090	ns
August -	January	-4.8270	0.008	**
	February	-0.4121	0.701	ns
	March	-3.6698	0.021	*
	April	-3.4731	0.026	*
	May	-8.3795	0.001	***
	June	-10.1272	0.001	***
	July	-4.6863	0.009	**
	September	-7.3753	0.002	**
	October	-5.818	0.004	**
	November	-3.9173	0.017	*
	December	-5.8427	0.004	**
September -	January	5.0130	0.007	**
	February	12.6747	0.000	***
	March	-0.2343	0.826	ns
	April	-0.6325	0.561	ns
	May	-4.1110	0.015	*
	June	-6.0104	0.004	**
	July	-1.4856	0.212	ns
	August	7.3753	0.002	**
	October	1.0000	0.004	**
	November	2.1893	0.017	*
	December	2.8284	0.004	**

Month		t-statistic	p value	Significance
October	- January	2.4495	0.070	ns
	February	8.0299	0.001	***
	March	-0.6022	0.580	ns
	April	-0.9258	0.470	ns
	May	-4.3301	0.012	*
	June	-6.0083	0.004	**
	July	-1.7961	0.147	ns
	August	5.8180	0.004	**
	September	-1.0000	0.374	ns
	November	1.2637	0.275	ns
	December	1.0000	0.374	ns
November	January	3.863	0.719	ns
	February	4.6628	0.010	**
	March	-1.2507	0.279	ns
	April	-1.4595	0.218	ns
	May	-4.8525	0.008	**
	June	-10.1272	0.001	***
	July	-2.3652	0.077	ns
	August	3.9173	0.017	*
	September	-2.1893	0.094	ns
	October	-1.2637	0.275	ns
	December	-1.0000	0.374	ns
December	January	2.064	0.108	ns
	February	9.8845	0.001	***
	March	-1.0151	0.367	ns
	April	-1.2649	0.275	ns
	May	-5.3759	0.006	**
	June	-7.4246	0.002	**
	July	-2.2283	0.090	ns
	August	5.8427	0.004	**
	September	-2.8284	0.047	*
	October	-1.0000	0.374	ns
	November	0.6768	0.536	ns

Appendix IId. Statistical data from Chapter 3. Probability values and t-statistics for Student's t-test in relation to monthly haemocyte counts

Comparison[1]	t-statistic[2]	p value[3]	Significance[4]
January - February	-0.7606	0.534	ns
March	-2.2649	0.030	ns
April	-5.0114	0.000	*
May	-3.3047	0.003	***
June	-4.9969	0.000	**
July	-5.4361	0.000	***
August	1.1269	0.269	***
September	-2.4214	0.022	ns
October	-2.4055	0.023	*
November	-2.9776	0.006	**
December	-2.4492	0.021	*
February - January	0.7606	0.453	ns
March	-1.1050	0.279	ns
April	-3.8476	0.001	***
May	-2.7162	0.011	*
June	-3.6616	0.001	***
July	-3.7570	0.001	***
August	1.6726	0.106	ns
September	-1.5679	0.128	ns
October	-1.2914	0.207	ns
November	-1.8073	0.081	ns
December	-1.4702	0.153	ns
March - January	2.2649	0.030	*
February	1.1050	0.279	ns
April	-3.4379	0.002	**
May	-2.2524	0.032	*
June	-3.2385	0.003	**
July	-3.4172	0.002	**
August	3.1202	0.004	**
September	-0.8156	0.422	ns
October	-0.3049	0.763	ns
November	-0.9635	0.344	ns
December	-0.6213	0.539	ns

[1]Months are February 1991-January 1992

[2]t- statistic for 28 degrees of freedom as given by Statworks statistical programme.

[3]The null hypothesis states that there is no difference between the mean values of the samples. Differences are significant at the 5% level if $p < 0.05$; 1% level if $p < 0.01$; 0.1% level if $p < 0.001$

[4]Asterisks indicate levels of significance: * = 0.05 % ** = 0 01 % *** = 0.001 %

n = 15 animals for each month.

	Month	t-statistic	p value	Significance
April -	January	5.0114	0.000	***
	February	3.8476	0.001	***
	March	3.4379	0.002	**
	May	-0.1012	0.920	ns
	June	0.5228	0.605	ns
	July	0.9167	0.367	ns
	August	5.4862	0.000	***
	September	2.0605	0.049	*
	October	3.0521	0.005	**
	November	2.4993	0.019	**
	December	2.5093	0.018	**
May -	January	3.3047	0.003	**
	February	2.7162	0.011	*
	March	2.2524	0.032	*
	April	0.1012	0.920	ns
	June	0.4533	0.654	ns
	July	0.6844	0.499	ns
	August	3.8027	0.001	***
	September	1.5562	0.131	ns
	October	2.0640	0.048	*
	November	1.7236	0.096	ns
	December	1.7948	0.083	ns
June -	January	4.9969	0.000	***
	February	3.6616	0.001	***
	March	3.2385	0.003	**
	April	-0.5228	0.605	ns
	May	-0.4533	0.654	ns
	July	0.3812	0.706	ns
	August	5.4638	0.000	***
	September	1.7210	0.096	ns
	October	2.7949	0.009	**
	November	2.1763	0.038	*
	December	2.1942	0.037	*

	Month	t-statistic	p value	Significance
July	- January	5.4361	0.00	***
	February	3.7570	0.001	***
	March	3.4172	0.002	**
	April	-0.9167	0.367	ns
	May	-0.6844	0.499	ns
	June	-0.3812	0.706	ns
	August	5.8030	0.000	***
	September	1.5718	0.127	ns
	October	2.8439	0.009	**
	November	2.1138	0.044	*
	December	2.1105	0.044	*
August	- January	-1.1269	0.269	ns
	February	-1.3726	0.106	ns
	March	-3.1202	0.004	**
	April	-5.4862	0.000	***
	May	-3.8027	0.001	***
	June	-5.4638	0.000	***
	July	-5.8030	0.000	***
	September	-3.1205	0.004	**
	October	-3.2222	0.003	**
	November	-3.6907	0.001	***
	December	-3.1935	0.003	**
September	- January	2.4214	0.022	*
	February	1.5679	0.128	ns
	March	0.8156	0.422	ns
	April	2.0605	0.049	*
	May	-1.5562	0.131	ns
	June	-1.7210	0.096	ns
	July	-1.5718	0.127	ns
	August	3.1205	0.004	**
	October	-0.5617	0.579	ns
	November	0.0674	0.947	ns
	December	0.2422	0.810	ns

	Month	t-statistic	p value	Significance
October	- January	2.4055	0.023	*
	February	1.2914	0.207	ns
	March	0.3049	0.763	ns
	April	-3.0521	0.005	**
	May	-2.0640	0.048	*
	June	-2.7949	0.009	**
	July	-2.8439	0.008	**
	August	3.2000	0.003	**
	September	-0.5617	0.579	ns
	November	-0.6216	0.539	ns
	December	-0.3387	0.737	ns
November	January	2.9776	0.006	**
	February	1.8073	0.081	ns
	March	0.9635	0.344	ns
	April	-2.4993	0.019	*
	May	-1.7236	0.096	ns
	June	-2.1763	0.038	*
	July	2.1138	0.044	*
	August	3.6907	0.001	***
	September	-0.0674	0.947	ns
	October	0.6216	0.539	ns
	December	0.2164	0.830	ns
December	January	2.4492	0.021	*
	February	1.472	0.153	ns
	March	0.6213	0.539	ns
	April	2.5093	0.018	*
	May	-1.7948	0.083	ns
	June	2.1942	0.037	*
	July	-2.1105	0.044	*
	August	-3.1935	0.003	**
	September	0.2422	0.810	ns
	October	-0.3387	0.737	ns
	November	0.2164	0.830	ns

Appendix IIe. Statistical data from Chapter 3. Probability values and t-statistics for Student's t-test in relation to monthly survival index data (February - November 1991)[1]

Comparison[2]		t-statistic[3]	p value[4]	significance[5]
February	- March	- 3.7796	0.019	*
	April	-4.9029	0.008	**
	May	-10.1193	0.001	***
	June	-12.7279	0.000	***
	July	-5.0138	0.007	**
	August	-0.3536	0.742	ns
	September	-13.4350	0.000	***
	October	-8.500	0.001	***
	November	-4.5962	0.010	**
March	- February	3.7796	0.019	*
	April	-0.6934	0.526	ns
	May	-2.000	0.116	ns
	June	-2.7440	0.052	ns
	July	-0.9439	0.399	ns
	August	-3.2585	0.031	*
	September	0.1890	0.859	ns
	October	0.5477	0.613	ns
	November	1.2005	0.296	ns
April	- February	4.9029	0.008	**
	March	0.6934	0.031	*
	May	-1.2005	0.296	ns
	June	-1.9445	0.124	ns
	July	-0.2747	0.797	ns
	August	4.2426	0.013	*
	September	1.1767	0.305	ns
	October	1.5119	0.206	ns
	November	2.1213	0.101	ns

[1]January and December data have been omitted because standard deviations exceeded means for each month.
[2]n=3 for all months.
[3]t- statistic for 4 degrees of freedom as given by Statworks statistics programme.
[4]The null hypothesis states that there is no difference between the mean values of the samples. Differences are significant at the 5% level if $p < 0.05$; 1% level if $p < 0.01$; 0.1% level if $p < 0.001$.
[5]Asterisks indicate levels of significance: * = 0.05 % ** = 0 01 % *** = 0.001 %

	Month	t - statistic	p value	Significance
May -	February	10.1193	0.001	***
	March	2.0000	0.116	ns
	April	1.2005	0.296	ns
	June	-1.000	0.374	ns
	July	0.8220	0.457	ns
	August	7.7500	0.001	***
	September	4.1110	0.015	*
	October	4.3301	0.012	*
	November	4.7500	0.009	**
June -	February	12.7279	0.000	***
	March	2.7440	0.052	ns
	April	1.9445	0.124	ns
	May	1.0000	0.374	ns
	July	1.5213	0.001	***
	August	9.3541	0.203	ns
	September	6.0104	0.004	**
	October	6.0083	0.004	**
	November	6.1470	0.004	**
July -	February	5.0138	0.007	**
	March	0.9439	0.399	ns
	April	0.2747	0.797	ns
	May	-0.8220	0.457	ns
	June	-1.5213	0.203	ns
	August	4.3948	0.012	*
	September	1.4856	0.212	ns
	October	1.7961	0.147	ns
	November	2.3664	0.077	ns
August -	February	0.3536	0.742	ns
	March	3.2585	0.031	*
	April	-4.2426	0.013	*
	May	-7.7500	0.001	***
	June	-9.3541	0.203	ns
	July	-4.3948	0.012	*
	September	-6.3640	0.003	**
	October	-5.0596	0.007	**
	November	-3.2071	0.033	*

Month	t - statistic	p value	Significance
September - February	13.4350	0.000	***
March	-0.1890	0.859	ns
April	-1.1767	0.305	ns
May	-4.1110	0.015	*
June	-6.0104	0.004	**
July	-1.4856	0.212	ns
August	6.3640	0.003	**
October	1.000	0.374	ns
November	2.1213	0.101	ns
October - February	8.500	0.001	***
March	-0.5477	0.613	ns
April	-1.5119	0.205	ns
May	-4.3301	0.009	**
June	-6.0083	0.004	**
July	-1.7961	0.147	ns
August	5.0596	0.007	**
September	-1.0000	0.374	ns
November	1.2649	0.275	ns
November February	4.5962	0.010	**
March	-1.2005	0.296	ns
April	-2.1213	0.101	ns
May	-4.7500	0.009	**
June	-6.1470	0.004	**
July	-2.3664	0.077	**
August	3.2071	0.033	*
September	-1.0000	0.374	ns
October	-1.2649	0.275	ns

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ANTIBACTERIAL ACTIVITY IN THE HAEMOCYTES OF THE SHORE CRAB, *CARCINUS MAENAS*

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The presence of antibacterial activity in the haemocytes of the shore crab, *Carcinus maenas* (L.) (Crustacea: Decapoda), was investigated using a selection of Gram-positive and Gram-negative bacteria from geographically diverse waters. Preliminary investigations into the relationship between this activity and the prophenoloxidase activating system (proPO) were also carried out. Antibacterial activity against both Gram-positive and Gram-negative organisms were found to reside exclusively in the granular haemocytes and eight of the twelve bacteria tested were susceptible to this effect. Additional studies, using *Psychrobacter immobilis* (= *Moraxella* sp.), revealed that the factor (or factors) responsible was 90% effective within 60 min and was also heat stable, independent of divalent cations, and non-lytic in character. Although antibacterial activity resides in the same cell population that carries the proPO system, there appears to be no relationship between antibacterial activity and phenoloxidase itself. Other components of the proPO system, however, may be involved.

INTRODUCTION

Numerous substances that kill foreign cells or micro-organisms have been recorded for invertebrates (see review by Ratcliffe *et al.*, 1985). Such factors are usually non-specific and their effects range from general disinfection, as with the pigment echinochrome A in echinoderms (Service & Wardlaw, 1984), to lysis of defined peptidoglycan bonds by lysozyme, found chiefly in insects (Powning & Davidson, 1973) and molluscs (McHenery *et al.*, 1979).

Within the arthropod group it is predominantly insects, crustaceans and chelicerates that have attracted research interest because of their economic or biomedical applications. The development of aquaculture has raised the status of crustaceans to one of considerable commercial significance and, for economic reasons, a scientific approach to the prevention and control of disease is of paramount importance. An integral part of this approach must be an understanding of the immune systems of the animals involved.

In crustaceans, as with other invertebrates, overall host defence largely depends on non-specific mechanisms such as phagocytosis, encapsulation, nodule formation, clotting and bacterial agglutination (Smith & Söderhäll, 1986). With the exception of bacterial agglutination, all are well documented (Smith & Chisholm, 1991). A complex enzyme system, the prophenoloxidase (proPO) activating system, present in granular haemocytes (Söderhäll & Smith, 1983), is also considered to be of importance in the host defence of these animals. Not only does the proPO system play a role in non-self recognition, but

additionally it provides opsonins, initiates capsule formation, participates in clotting and mediates in haemocyte co-operation (Smith & Söderhäll, 1986; Söderhäll *et al.*, 1990). Antibacterial activity in crustacean haemolymph, as distinct from agglutination, phagocytosis or encapsulation, has been described relatively infrequently and appears to be an area of research which has been largely overlooked (see review by Smith & Chisholm, 1991). Where such activity has been identified it has, in general, been ascribed to a 'factor' or 'factors' which remain poorly characterized.

Studies have tended to focus on commercially important species (for example crabs and lobsters) in relation to responses to pathogens such as *Aerococcus viridans* var. *homari* (=*Gaffkya homari*) (Cornick & Stewart, 1968a,b; Stewart & Zwicker, 1972). A few reports exist with respect to antibacterial activity in other crustaceans and in these the test organisms have usually been Gram-negative bacteria, including isolates from the gut of the host (Evans *et al.*, 1968, 1969; Weinheimer *et al.*, 1969; Mori & Stewart, 1978). Typically plasma or serum preparations have been used to challenge the test organisms, whereas, in the series of experiments described here, the specific intention has been to investigate the antibacterial properties of *Carcinus maenas* haemocytes in isolation from the humoral components of haemolymph. We also describe preliminary investigations designed to explore the possibility of a relationship between antibacterial activity and the proPO activating system, the terminal products of which are known to have anti-fungal properties (Nyhlén & Unestam, 1980; Söderhäll & Ajaxon, 1982).

MATERIALS AND METHODS

Animals

Specimens of the common shore crab, *Carcinus maenas*, were collected from St Andrews Bay, kept in a flowing sea-water aquarium and fed twice weekly on minced fish. Only healthy male intermoult animals (7-10 cm carapace width) were used for experimental purposes and each animal was subjected to a single bleed.

Bacteria

Twelve strains of Gram-positive and Gram-negative marine bacteria from geographically diverse waters were used for *in vitro* tests of antibacterial activity in *Carcinus maenas* haemocytes and are listed in Table 1. The majority of the organisms had distinctive colony formations (Table 1), and all were cultured in Bacto Marine Broth 2216 or on Bacto Marine Agar 2216 (Difco Labs).

The three Antarctic isolates, designated BS 66, BS 68 and BS 78, were raised to log-phase growth in marine broth at 18°C for 12-18 h. The temperate water bacteria (*Psychrobacter immobilis*, *Pseudomonas* 3-1-1, *Photobacterium phosphoreum*, virulent and avirulent strains of *Aerococcus viridans* var. *homari* (= *Gaffkya homari*), *Planococcus citreus* and two serotypes of *Vibrio anguillarum*) were raised at 20°C for 24 h. *Pseudomonas* 1-1-1 (an agar-digester) was cultured on marine agar slopes and harvested by washing the slopes with sterile 3·2% NaCl (pH 7·0). Culture of *A. viridans* (virulent) was facilitated by supplementing marine broth with lobster serum (1 ml per 50 ml broth). The serum was prepared using,

Table 1. *Bacteria used in assays for antibacterial activity in* Carcinus maenas *haemocytes*

Bacteria	Gram reaction	Incubation temp. for marine agar plates (°C)[6]	Colony appearance
Psychrobacter immobilis (NCIMB[1] 308)	negative	32	cream
Photobacterium phosphoreum (NCIMB[1] 844)	negative	20	white, luminescent
Pseudomonas 3-1-1 [2]	negative	32	pink
Pseudomonas 1-1-1 [2]	negative	20	black, agar digesting
Vibrio anguillarum 8575 (Serotype I)[3]	negative	20	orange-yellow
Vibrio anguillarum 8587 (Serotype II)[3]	negative	20	orange-yellow
BS 78[4]	negative	15	cream
Planococcus citreus (NCIMB[1] 1493)	positive	32	orange
Aerococcus viridans var. *homari* [5] virulent	positive	20	white
Aerococcus viridans var. *homari* [5] avirulent	positive	20	white
BS 68[4]	positive	15	buff
BS 66[4]	positive	15	yellow

[1]NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland. [2]*Pseudomonas* 3-1-1 and *Pseudomonas* 1-1-1 are marine isolates from the west coast of Scotland. [3]*Vibrio anguillarum* provided by the Institute of Aquaculture, University of Stirling, Scotland. [4]Marine Antarctic isolates from surface waters in Bransfield Strait, from The British Antarctic Survey. [5]*Aerococcus viridans* var. *homari* from the Department of Agriculture and Fisheries, Aberdeen, Scotland. The virulent strain had been passaged through lobsters and the avirulent strain had been maintained on agar slopes. [6]Temperate water bacteria were incubated for 48-72 h, Antarctic strains for 3-5 d.

essentially, the method described for the shore crab by Smith & Ratcliffe (1978). No serum supplement was used when raising *A. viridans* (virulent or avirulent) on marine agar plates.

Bacteria were prepared by centrifugation at 1,900*g* for 10 min, washed twice in sterile 3·2% NaCl, and finally resuspended in sterile 3·2% NaCl. These suspensions were calibrated, using spread plates, and were routinely standardized to an absorbance of 0·5 at 570 nm. Serial dilutions were performed in sterile 3·2% NaCl to give concentrations of between $2x10^6$ and $5x10^6$ ml^{-1} before use in antibacterial assays.

Preparation of haemocyte lysate supernatants (HLS)

Haemolymph in sterile marine anticoagulant (pH 4·6) was obtained aseptically as described in Söderhäll & Smith (1983), using 5-7 crabs for each HLS preparation. Samples were pooled, centrifuged at 1,900*g* (10 min at 4°C) and the resultant haemocyte pellet was washed once in citrated cacodylate buffer (CAC), pH 7·0 (45 mM sodium chloride, 10 mM sodium cacodylate and 100 mM trisodium citrate). After further centrifugation at 1,900*g* (10 min at 4°C), the cell pellet was homogenized for 10 min in 5 ml sterile *Carcinus* saline, pH 7·4, (modified from Smith & Ratcliffe (1978) to contain 20 mM $CaCl_2.6H_2O$ and no magnesium), using an ice-cold, pyrogen-free, glass piston homogenizer. The homogenate was centrifuged in a sterile polycarbonate tube at 40,000*g* (20 min at 4°C) and the supernatant, designated HLS, was stored on ice for no more than 15 min before use in antibacterial assays.

Preparation of separated cell lysate supernatants

Haemocyte populations were separated in 10 ml of 60% Percoll (Pharmacia, Uppsala, Sweden) in 3·2% NaCl as described in Söderhäll & Smith (1983). Hyaline or granular cell

fractions from five crabs were pooled into 20 ml CAC buffer (pH 7·0) in polycarbonate tubes before centrifugation at 1,900*g* (10 min at 4°C). Hyaline cell lysate supernatants (HyLS) or granular cell lysate supernatants (GLS) were then prepared, each in 2 ml *Carcinus* saline, as described above for HLS. Ice-cold buffers were used during the preparation of all cell lysate supernatants.

Purity of hyaline cell fractions was checked, using the rapid drop-assay given in Söderhäll & Smith (1983) which utilizes phenoloxidase as a marker for the granular cells. Only those fractions exhibiting negative or negligible phenoloxidase activity were used to prepare HyLS samples.

Plasma preparation

A 2-ml sample of haemolymph was obtained aseptically in the absence of anticoagulant and was centrifuged immediately at 600*g* in a sterile polycarbonate tube (4°C for 15 min). The plasma supernatant was then transferred to an ice-cold, sterile polycarbonate tube and used straight away.

Protein determination

Protein was determined for each lysate supernatant or plasma preparation using the method described by Bradford (1976). Bovine serum albumin (BSA) was used as standard. Unless otherwise indicated HLS protein levels were adjusted to between 1·5 and 1·8 mg ml^{-1} for all experiments.

Assay for antibacterial activity in HLS

The standard assay involved incubation of 900 µl of HLS with 100 µl of bacterial suspension in sterile Eppendorf tubes to give a final concentration of 2-5x10^5 bacteria ml^{-1}. Equal parts of sterile marine broth and sterile *Carcinus* saline were used as substitute for HLS in control tubes. At intervals 100 µl aliquots of test or control solutions were serially diluted (1:10 in sterile 3·2% NaCl, pH 7·0) to give countable colonies, and 100 µl of the last two dilutions were plated out in triplicate on marine agar. Plates were incubated for 48-72 h at the temperatures indicated in Table 1.

Antibacterial activity was recorded as a survival index (SI) as given in Wardlaw & Unkles (1978):

$$SI = \frac{\text{Number of colonies at time, t}}{\text{Number of colonies at time, 0}} \times 100 \qquad (1)$$

Hence, an SI greater than 100 indicates growth and one of less than 100 indicates antibacterial activity.

Investigations into antibacterial activity in HLS

Several characteristics of the antibacterial factor in *Carcinus maenas* HLS were investigated, namely the spectrum of activity, divalent cation dependence, thermal stability and titre. Spectrum of activity was studied using the 12 bacteria listed in Table 1. After challenge with HLS, aliquots of experimental and control solutions were removed at 0,

1, 2, 4 and 6 h and SI values recorded.

Two HLS samples were used in each test for divalent cation dependence, one prepared in 2 ml calcium-depleted *Carcinus* saline and the other in 2 ml *Carcinus* saline containing 20 mM calcium. Both preparations were derived from a single pooled sample of haemolymph in anticoagulent which had been split prior to the first centrifugation.

Heat stability was assessed using 10 ml HLS prepared from the pooled haemolymph of 10-12 crabs. Two-millilitre aliquots were transferred to sterile bijoux and one sample was retained on ice until needed. Four samples were heat-treated at 50, 60 or 70°C for 20 min or at 100°C for 30 min, cooled rapidly and stored on ice until all samples were ready for use.

Freeze-thaw stability was investigated using 12 ml of HLS prepared from pooled samples from 12 animals and 900-µl aliquots transferred to sterile Eppendorfs. Two samples were retained for immediate use and the rest were stored either at -20°C or -70°C, for one week, one month or three months. After these periods samples were thawed at 20°C and, prior to testing, were agitated vigorously to dislodge any protein material which may have adhered to the walls of the tubes.

Concurrent assays for phenoloxidase activity were performed on each sample of HLS used in the heat and freeze-thaw stability tests, following the method described in Söderhäll & Smith (1983). Lipopolysaccharide (LPS) (from *E. coli* 0111:B4, phenolic extract, Sigma, Poole, Dorset), 1 mg ml^{-1} in *Carcinus* saline, was used as elicitor and *Carcinus* saline was substituted for LPS in the buffer controls. Enzyme activity was expressed as units per mg of protein where one unit represents the change in absorbance at 490 nm per minute.

For titre of activity, HLS preparations were standardized to an initial protein concentration of 1·8 mg ml^{-1}. After serial ten-fold dilutions to 10^{-4} in *Carcinus* saline, 900 µl of each dilution were tested for antibacterial activity. Titre is given as the reciprocal of the highest dilution at which antibacterial activity was detected.

Location of antibacterial activity

Assays were carried out using GLS and HyLS, to confirm the results of previous preliminary investigations into the location of antibacterial activity in different cell types (Söderhäll & Smith, 1986a). Protein levels in GLS and HyLS were approximately an order of magnitude lower than than those in HLS and, to compensate for this, the initial concentration of the bacterial inoculum was reduced by a factor of ten. Additionally, plasma from five individual crabs was tested for antibacterial activity and also phenoloxidase activity, using the appropriate buffer substitutes in controls.

Experiments relating to thermal stability, divalent cation dependence, titre and location of activity were carried out at 20°C with an incubation time of 4 h.

Lysis assays

Turbimetric studies were used to determine whether changes in SI of *Psychrobacter immobilis* were due to lysis. Five hundred microlitres of HLS and 500 µl of bacteria (absorbance 0·5 at 570 nm; initial concentration, $2x10^7$ bacteria ml^{-1}) were incubated together at 20°C in the experimental tubes, with equal parts of marine broth and *Carcinus*

saline substituted for HLS in controls. Turbidity changes were recorded initially at 10, 20, 30, 60, and 120 s, after which the tubes were incubated for 4 h, with further turbidity readings recorded at the end of this period. At the end of the experiment the numbers of viable bacteria in test and control solutions were determined by spread plates in the usual way. Alterations in turbidity were recorded as a percentage of the initial turbidity reading at 10 s and SI values were calculated from the spread plates as above.

Bacterial lawn assays were carried out to confirm the observations made with turbidity studies. Lawns were prepared with soft agar overlays, using a mixture of 2·5 ml marine agar and 2·5 ml of an 18-h broth of *P. immobilis*. After incubation at 20°C for 24 h, wells were prepared in the lawns using a sterile no. 2 cork-borer. Fifty microlitres of HLS or control saline were added to the wells and the plates allowed to incubate overnight at 20°C.

RESULTS

Spectrum of activity

In control tubes all 12 species of bacteria had SI values in excess of 100 over the incubation period of 6 h (Figures 1A-E & 2A-G). Of the five Gram-positive organisms tested, two, BS 66 and BS 68 (the Antarctic bacteria), were particularly sensitive to the antibacterial effect, with SI values approximating to zero after 6 h (Figure 1A,B). These

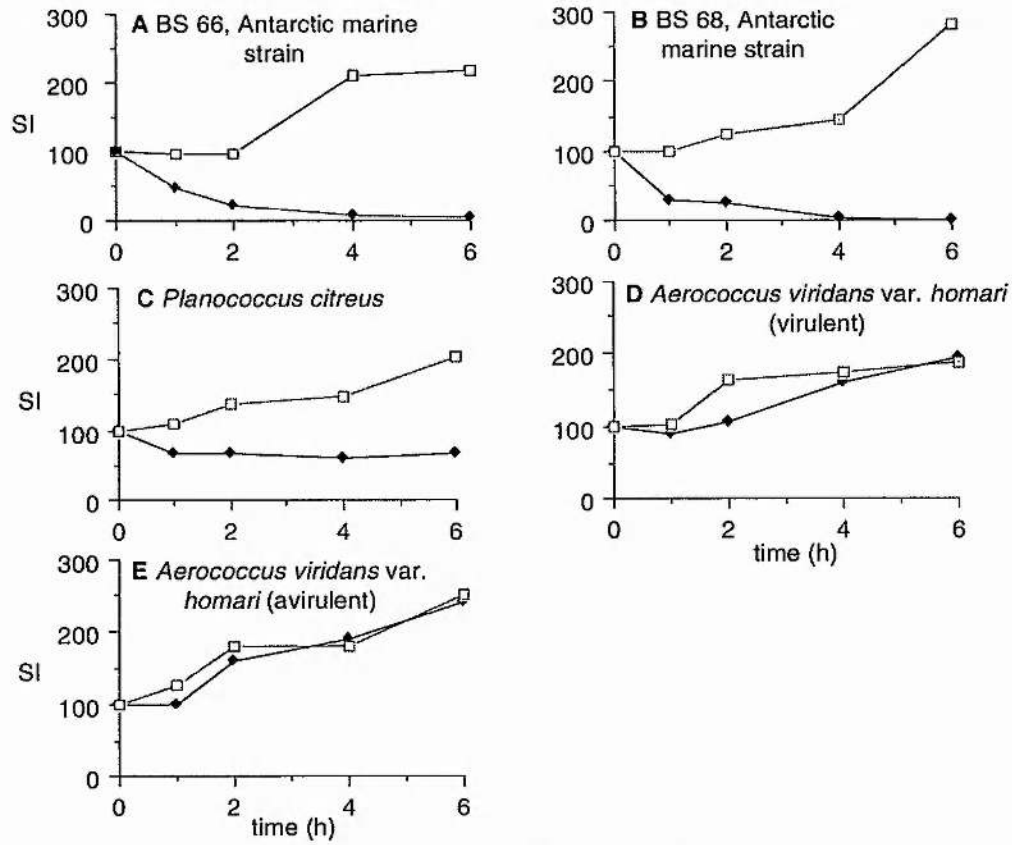

Figure 1. Effect of haemocyte lysate supernatant on the survival index (SI) of Gram-positive bacteria. Results shown relate to individual experiments. Replicate experiments all showed the same trends. Controls comprised equal parts of marine broth and *Carcinus* saline as substitute for HLS (haemocyte lysate supernatant, prepared as described in Materials and Methods). □, control; ◆, HLS.

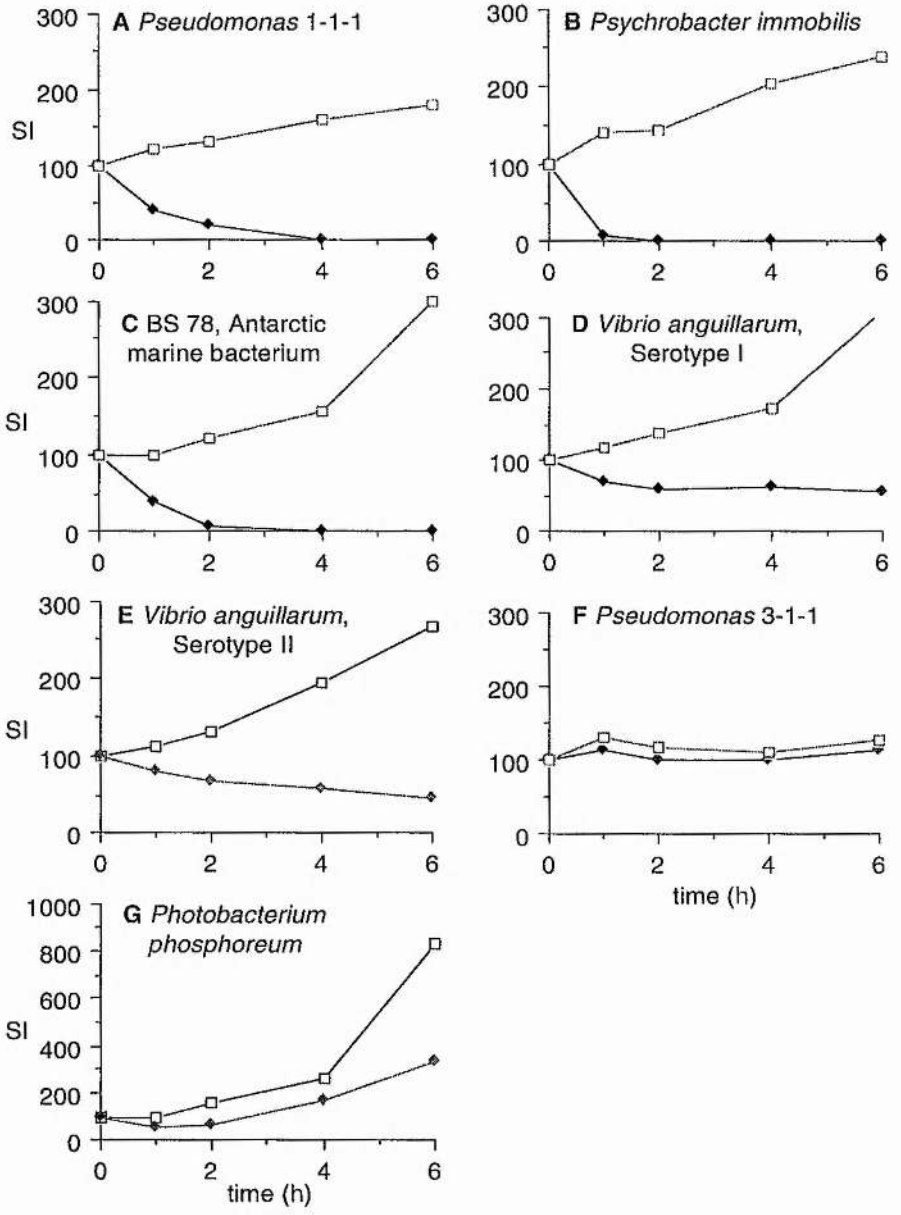

Figure 2. Effect of haemocyte lysate supernatant on the survival index (SI) of Gram-negative bacteria. Results shown relate to individual experiments. Replicate experiments all showed the same trends. Controls comprised equal parts of marine broth and *Carcinus* saline as substitute for HLS (haemocyte lysate supernatant, prepared as described in Materials and Methods). □, control; ◆, HLS.

organisms also responded rapidly within the first hour of challenge, as indicated by the slope of the curves in Figure 1A,B. Another Gram-positive bacterium, *Planococcus citreus*, also exhibited a reduction in SI (but to 67·4 after 6 h), and compared with BS 66 and BS 68, the response was slower (Figure 1C). By contrast, both virulent and avirulent strains of *Aerococcus viridans* flourished in the presence of HLS, with final SI values of 190 and 240 respectively (Figure 1D,E), growth patterns closely following those of their respective controls.

Five of the seven Gram-negative bacteria were sensitive to the effect of HLS, but to different degrees. *Pseudomonas* 1-1-1 (Figure 2A), *Psychrobacter immobilis* (Figure 2B) and

BS 78 (Figure 2C) produced SI values approaching zero within 4 h, whilst the two serotypes of *Vibrio anguillarum* (Figure 2D,E), gave an intermediate result, with survival indices of 57 for serotype I (Figure 2D) and 45 for serotype II (Figure 2E) after 6 h. Of the two remaining Gram-negative bacteria, *Pseudomonas* 3-1-1 gave a response that was similar to that of the corresponding control with an SI of 112, indicating slight growth over 6 h (Figure 2). On the other hand, *Photobacterium phosphoreum* grew in the presence of HLS over the same time interval (to give an SI of 340) but to a lesser extent than the associated control where the SI was over 830 (Figure 2G).

Overall, an antibacterial effect was found to operate against eight of the twelve species challenged with HLS from *Carcinus maenas*. All data presented relate to individual representative experiments and the same trends were noted in all replicated investigations.

Requirement for divalent cations

Antibacterial activity was independent of calcium ions. The bacterial response appeared identical in both calcium depleted and calcium supplemented HLS preparations, with SI values of almost zero attained within 1 h. Furthermore, the antibacterial effect must be independent of magnesium ions since HLS is routinely prepared in a magnesium-free buffer.

Thermal stability

Table 2 shows a progressive increase in SI with increasing temperature. After 4 hours' incubation SI values of <0·001, 4·7 ±1·1, 4·1 ±1·4 and 37·1 ±2·5 were recorded for samples heat-treated at 50, 60, 70 and 100°C respectively. These values represent a gradual loss of antibacterial activity; nevertheless, an SI of 37 after treatment to 100°C indicates retention of ~60% of the activity recorded for fresh HLS. With respect to the possible relationship between antibacterial activity and phenoloxidase activity it was observed that whilst increasing temperature up to 60°C resulted in a slight decline in antibacterial effect (SI 4·7 compared with SI <0·001 for fresh HLS), corresponding phenoloxidase assays showed an enhancement of enzyme activity (Table 2). However, at 70°C phenoloxidase activity was reduced by 50% and, after heating to 100°C, was negligible (Table 2), although antibacterial activity at these temperatures was still measurable.

Table 2. *Antibacterial activity and phenoloxidase activity in heat-treated HLS*

	P. immobilis Survival index		Phenoloxidase activity (units mg^{-1} protein)[1]	
Treatment	1 hour	4 hours	LPS treated[2]	Buffer control[3]
Fresh	1.9 ± 0.9	< 0.001	0.04 ± 0.01	0.02 ± 0.01
50°C (20 min)	5.3 ± 3.1	<0.001	0.10 ± 0.04	0.04 ± 0.02
60°C (20 min)	22.0 ± 6.0	4.7 ± 1.1	0.14 ± 0.01	0.06 ± 0.03
70°C (20 min)	51.7 ±11.8	4.1 ± 1.4	0.07 ± 0.01	0.05 ± 0.01
100°C (30 min)	57.0 ±10.9	37.1 ± 2.5	< 0.0001	< 0.0001
Control[4]	130.9 ±23.7	201.9 ±42.7		

Values are means ±SD (N=3). [1]Phenoloxidase activities are calculated from absorbances read at 490 nm. [2]HLS preparations were pre-incubated with LPS (1 mg ml^{-1}) for 20 min before addition of L-dopa (3 mg ml^{-1}). [3]Controls for the phenoloxidase assay contained *Carcinus* saline as substitute for LPS. [4]Controls for the antibacterial activity assay contained equal parts of *Carcinus* saline and marine broth as substitute for HLS.

Table 3. *Antibacterial activity and phenoloxidase activity in HLS freeze-treated to -70°C*

Treatment	*P. immobilis* Survival index[1] 1 hour	4 hours	Phenoloxidase activity (units mg^{-1} protein)[2] LPS treated[3]	Buffer control[4]
Fresh	38.6 ± 2.0	4.2 ± 1.9	0.106 ± 0.060	0.010 ± 0.005
1 week	19.2 ± 9.3	4.8 ± 4.2	0.127 ± 0.015	0.009 ± 0.002
1 month	27.4 ± 2.3	4.8 ± 2.4	0.119 ± 0.030	0.013 ± 0.003
3 months	4.8 ± 2.7	< 0.001	0.031 ± 0.003	0.023 ± 0.004

Values are means ±SD (N=3). [1]SI values are for experimental tubes only. Control SI values at both 1 h and 4 h exceeded 100 for all treatments. Control tubes contained equal parts of *Carcinus* saline and marine broth as substitute for HLS. [2]Phenoloxidase activities are calculated from absorbances read at 490 nm. [3]HLS preparations were pre-incubated with LPS as elicitor (1 mg ml^{-1}) for 20 min before addition of L-dopa (3 mg ml^{-1}). [4]Controls for the phenoloxidase assay contained *Carcinus* saline as substitute for LPS.

With respect to freeze-stability Table 3 shows that after 1 h incubation the antibacterial effect of HLS was markedly enhanced following storage at -70°C for three months; the SI for *P. immobilis* challenged with freeze-thawed HLS was 4·8 whereas an SI of 38·6 was recorded after challenge with fresh material. After 4 h the same samples gave SI values of almost 0 and 4·2 respectively. Enhanced antibacterial activity was also seen at 1 h incubation with intermediate periods of storage at -70°C, but the effect was less pronounced (see Table 3 for data). Similar results were obtained from material frozen at -20°C (results not shown). Again, associated phenoloxidase assays failed to reveal any relationship between enzyme activity and antibacterial effect (Table 3). Approximately 30% loss of enzyme activity occurred after three months' storage at -70°C, although there was no parallel increase in SI of *P. immobilis* (Table 3).

Table 4. *Titre of antibacterial activity against* Psychrobacter immobilis

Dilutions	Protein concentration(ml^{-1} HLS)	Survival index at 4 h
Undiluted[1]	1.8 mg	2.8 ± 1.3
10^{-1}	0.18 mg	4.1 ± 2.5
10^{-2}	0.018 mg	13.4 ± 1.2
10^{-3}	18 µg	43.9 ± 21.0
10^{-4}	1.8 µg	61.3 ± 11.6
Control[2]		238.0 ± 95.0

Values are means ±SD (N=3). [1]HLS preparations were standardized to a protein concentration of 1·8 mg ml^{-1} before dilution. [2]Controls contained equal parts of *Carcinus* saline and marine broth as substitute for HLS.

Titre

The antibacterial factor present in *Carcinus maenas* haemocytes was found to be active at high titre, 10^4 (a protein concentration of about 2 µg ml^{-1}) (Table 4). Although the trend was for SI to increase (*i.e.* antibacterial activity to weaken) with increasing titre, it was still possible to detect a reduction of SI, compared with the control, at a titre of 10^4 after 4 h incubation.

Location of antibacterial activity

Comparison of the GLS, HyLS and HLS revealed that antibacterial activity resides exclusively in the GLS preparation, with the effect being almost identical to that found

Table 5. *Effect of separated cell lysate supernatants and HLS on* Psychrobacter immobilis

Cell preparation	Survival index at 4 h[1]
HLS[2]	3.8
HyLS[3]	304.8
GLS[4]	5.3
Control[5]	267.8

[1]Values are results from a typical experiment. The same trend was observed in all replicated experiments (N=3). [2]HLS, haemocyte lysate supernatant from mixed cells. [3]HyLS, hyaline cell lysate supernatant, prepared as described in Materials and Methods. [4]GLS, granular cell lysate supernatant, prepared as described in Materials and Methods. [5]Contols contained equal parts of *Carcinus* saline and marine broth as substitute for HLS.

in HLS (Table 5). By contrast, the bacteria thrived in HyLS (SI of ~300 after 4 h) (Table 5).

An SI of 52·6 was recorded after incubation of *Psychrobacter immobilis* in plasma prepared by centrifugation of whole haemolymph at 600*g* for 15 min (Table 6). These plasma samples also showed low levels of phenoloxidase activity (Table 6). To clarify whether the method of plasma preparation may have influenced SI values, additional experiments were carried out using whole haemolymph centrifuged at 500*g* or 1,900*g* for 15 min. As Table 6 shows, increasing the centrifugal force appeared to affect the antibacterial activity in the plasma sample. The 500*g* plasma gave an SI of 201 whereas the 1,900*g* plasma gave an SI of 0·5 (Table 6). Phenoloxidase activity was also affected; an approximate ten-fold increase in activity was observed in the 1,900*g* plasma when compared with the 500*g* plasma (Table 6).

Table 6. *Effect of plasma on the survival index of* Psychrobacter immobilis

Treatment[1]	Survival index at 4 h	Phenoloxidase activity (mg^{-1} protein)[3]
500*g*	201.0 ± 28.0	0.008 ± 0.001
600*g*	52.6 ± 13.2	0.014 ± 0.002
1900*g*	0.5 ± 0.1	0.090 ± 0.02
Control[2]	493.0 ± 85.0	

Values are means ±SD (N=5). [1]Haemolymph was centrifuged for 15 min as described in Materials and Methods. [2]Controls contained marine broth as substitute for plasma. [3]Phenoloxidase activity was measured at 490 nm, using LPS (1 mg ml^{-1}) as elicitor.

Lytic activity

No lytic activity against *P. immobilis* was detected by either turbimetric assay or lysis plates. Despite this, assays of antibacterial activity by the spread plate method showed a loss of viable organisms from test solutions over the 4-h period. Bacterial lawns showed no evidence of clearing, although some blackening of the agar was observed around the experimental wells, probably due to melanin deposition as a consequence of phenoloxidase activation in the HLS.

DISCUSSION

Antimicrobial factors represent one component of the immune response and have been described for a number of invertebrate groups (see review by Ratcliffe *et al.*, 1985).

Within the Crustacea, observations are restricted to about seven species, one of which, *Callinectes sapidus*, shows anti-viral activity (McCumber & Clem, 1977) and another, *Pacifastacus leniusculus* (Nyhlén & Unestam, 1980), anti-fungal activity. For the rest (*Panulirus argus*, *Panulirus interruptus*, *Homarus americanus*, *Penaeus monodon* and *Carcinus maenas*) none of the recorded factors has been fully characterized and none has been purified (see review by Smith & Chisholm, 1991).

The present study, using *C. maenas*, demonstrates that an antibacterial factor (or factors) exists in the haemocytes and is absent from plasma. The antibacterial effect operates against both Gram-positive and Gram-negative organisms, including those from geographically remote waters, and is rapid in action. We also show the factor(s) to be independent of calcium ions, heat stable and active at high titre.

Amongst the Gram-positive strains tested, the strongest antibacterial activity, as evidenced by low survival indices, was against the two Antarctic bacteria (BS 66 & BS 68). This contrasts sharply with the apparent lack of antibacterial effect on both virulent and avirulent strains of *Aerococcus viridans*. Similarly, the survival indices of the the Gram-negative bacteria, *Psychrobacter immobilis*, *Pseudomonas* 1-1-1 and BS 78 were markedly depressed when challenged with HLS, whereas those of *Photobacterium phosphoreum* and *Pseudomonas* 3-1-1 were not. This variability in response by both Gram-negative and Gram-positive forms suggests that there may be particular characteristics of some organisms which render them more susceptible than others to the antibacterial factor in HLS.

The resistance of both virulent and avirulent strains of *A. viridans* to challenge with HLS preparations is compatible with the mild pathogenicity that this bacterium is known to have for some species of crab (Cornick & Stewart, 1968b, 1975). It has been shown in lobsters that haemolymph is an excellent nutrient medium for *A. viridans* and, despite being phagocytosed and encapsulated, the bacterium is capable of continued growth *in vivo* (Cornick & Stewart, 1968a,b), to the detriment of the host.

The final SI values of the Antarctic strains in control solutions (all in excess of 200) indicate that, for the duration of the experiment, the incubation temperature of 20°C was not incompatible with growth. Indeed, many polar bacteria are capable of growth at this temperature (Herbert, 1986).

Little is known about the biochemical and biophysical properties of antibacterial factors in crustaceans. A factor in one species, *Panulirus argus*, which has been more fully investigated, shows no requirement for divalent cations (Evans *et al.*, 1968) and an equivalent finding is reported in the present study for *C. maenas*.

The freeze-stability of the antibacterial factor reported here also occurs in *P. argus* (Evans *et al.*, 1968). However, the reason for enhancement of antibacterial activity in *Carcinus* HLS, occurring after prolonged freezing, remains enigmatic. For crustaceans as a group there has been no attempt to define the mechanism(s) by which antibacterial factors are regulated, so whether our observations result from a loss of modulators or are due to some separate effect, such as conformational change of bioactive factors, is unknown.

With respect to heat stability, the antibacterial factor in *C. maenas* haemocytes appears stable to 70°C and has measurable residual activity after heating to 100°C. The factor or factors in *C. maenas* are thus more robust than those present in *P. argus* (Evans *et al.*, 1968) and *P. interruptus* (Evans *et al.*, 1969) in which the antibacterial effect is lost at 65°C.

Antibacterial activity in *C. maenas* can also be measured at high titre, suggesting that the factor responsible is potent.

The mode of action of the antibacterial factor(s) is unclear but it seems unlikely that our observations can be attributed to agglutination. The features of heat stability, activity at high titre and no requirement for divalent cations, distinguish antibacterial activity *per se* from agglutination in crustaceans. In general, the latter response is inactivated at 60°C, operates at low titre, and is dependent on divalent cations (see review by Smith & Chisholm, 1991). It should also be noted that for *C. maenas*, although Smith & Ratcliffe (1978) have reported the presence of haemagglutinins, bacterial agglutinins have not, so far, been identified in this animal.

Recent observations by Fenouil & Roch (1991) have revealed that lysozyme occurs in the haemolymph of freshwater crayfish, but we have found no evidence to suggest that the antibacterial activity reported for *Carcinus maenas* haemocytes is lytic in nature and, as yet, have found no evidence of lysozyme (unpublished observations).

The fact that antibacterial activity resides in the granular cells, which also contain the proPO system, raises the question of whether the appearance of this activity is dependent on activation of the proPO system. As yet we have been unable to identify an inhibitor of the proPO system which does not also inhibit bacterial growth. An alternative approach would be to investigate the level of proPO activation in HLS by the different strains of test bacteria. However, this presents a different set of problems, namely that of purifying bacterial cell wall products, from all strains, for phenoloxidase assays.

Although the relationship of the proPO system to antibacterial activity in *C. maenas* haemocytes remains equivocal, it is clear that phenoloxidase itself is not responsible for the antibacterial effect, since reduction of bacterial number in test solutions persists even when phenoloxidase activity has been lost by heating, or has been substantially reduced by freezing. However, the proPO system is a complex cascade of enzymes, which generates several bioactive molecules, including peptides (Söderhäll & Smith, 1986b) which could contribute to the antibacterial effect in this animal. Peptides are known to have antibacterial properties in insects and other animals (Boman, 1991), so an antibacterial role for other factors in the proPO system of *C. maenas* cannot be ruled out.

The absence of antibacterial activity in plasma, reported in this study, supports a previous finding by White *et al.*, (1985). Our observations were heavily dependent on the method of plasma preparation. Crustacean granular cells exocytose readily in the presence of endotoxins *in vitro* (Smith & Söderhäll, 1983) and furthermore, from the results of the present study, it seems that the extent to which these cells lyse or exocytose is affected by the centrifugal force they experience during plasma preparation. Contamination of our plasma preparations by granular cell products, as marked by phenoloxidase levels, clearly influenced the results of antibacterial assays, giving low SI values for the test organisms.

Work is in progress to ascertain the biochemical identity of the granular cell products responsible for antibacterial activity in *C. maenas* haemocytes. So far we do not know whether our observations can be ascribed to a single factor or whether they reflect the combined effect of a battery of factors. Factors such as cecropins and defensins represent unique families of antibacterial molecules and have been described in animals as diverse

as lepidopteran insects (Boman, 1986), amphibians (Zasloff, 1987) and pigs (Lee *et al.*, 1989). They are known to act rapidly against both Gram-positive and Gram-negative bacteria, and the advantages that these molecules confer on their invertebrate hosts are discussed eloquently in a recent paper by Boman (1991). Full characterization of the factor in *C. maenas* may further contribute to our understanding of the phylogeny of defence molecules in invertebrates.

The ability of an animal to mount an effective immune response is an essential prerequisite for continued survival, reproduction and evolutionary success and the presence of a rapidly effective antibacterial factor in crustacean haemocytes may have wider implications in the context of aquaculture with its associated problems of stress-related disease. To date no assessments have been made as to the potential therapeutic or prophylactic applications for crustacean antibacterial factors. It would be interesting to determine, however, whether the level of activity of such a factor could be employed as a convenient measure of immunocompetence or stress in these animals.

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Review Article

Non-cellular immunity in crustaceans

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Following the tradition established for vertebrates, the host defences of invertebrates are often categorised as either humoral or cellular, i.e. mediated by factors in the plasma or brought about by activities of intact blood cells. This distinction, while convenient, may be inappropriate for some invertebrate groups and may cloud our understanding of the defence processes in lower animals. The present review considers the so-called humoral strategies in crustaceans and attempts to assess the current state of knowledge in this area. In addition, we explore the validity of separating cellular from non-cellular defence reactivity in these invertebrates. The presence, biological activity, biochemical features and origin of the agglutinins, killing factors, lysins, precipitins, cytokine-like molecules and clotting agents in the blood of various crustacean species are described, and their relationship to the cellular defences discussed. It is concluded that while some of these factors appear to represent components of a genuine humoral defence system, others are (or may be) derived from the circulating haemocytes, with several operating effectively only in conjunction with the cellular network. In view of the central role played by the cells in mediating immune capability in crustaceans we suggest that the acquisition of the complex clotting pathway in arthropods might have provided the means for the development of the cellular messenger systems characteristic of crustacean immunity. However, much still needs to be done to clarify the status, biological roles and source of the 'humoral' factors in crustaceans, particularly with respect to their biochemical identity and role in protection of the host. Until a better understanding of these defences is achieved, the prospects for the development of effective vaccines for commercially valuable species will be limited and the potential of the constituent factors as useful indicators of environmental stress will remain obscure.

Key words: humoral immunity, invertebrate immunity, crustaceans, agglutinins, lysins, microbicidins, recognition, phenoloxidase.

1. Introduction

From the inception of immunology as a scientific discipline, marked by Metchnikoff's discovery of phagocytosis in invertebrates, the subject has been characterised by the separation of immune reactivity into cellular or humoral components. Originally, this distinction reflected opinions about the nature of non-self recognition, with the phenomenon being variously, and often controversially, explained in terms of the cells that mediate host defence or as the

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function of specific molecules in the serum. For vertebrates, this mechanistic dichotomy to all intents and purposes has now been resolved, although the terms have tended to persist largely for reasons of convenience. At its peak, the cellular *v.* humoral debate served to accelerate our understanding of antigen specificity and lymphocyte activity in higher animals, but it diverted attention from Metchnikoff's original questions about innate recognition and defence responses, leaving comparative aspects of immunology somewhat neglected.

In recent years, the development of aquaculture plus an increasing awareness of the significance of invertebrate vectors in parasitic disease and the desire to find suitable non-mammalian hosts for experimentation, has resulted in a resurgence of interest in comparative immunology. Many of these investigations are directed at addressing fundamental issues about the nature of recognition and blood cell activation in lower animals, reviving Metchnikoffian ideas with the application of modern molecular techniques. Much is being revealed about the biochemical events involved in non-specific recognition in different phyla and also about the evolution of immunity within the animal kingdom. However, following the tradition established for vertebrates, immune processes in invertebrates are still broadly categorised as either cellular or humoral, despite wide acceptance that invertebrates differ significantly from vertebrates in lacking defined immunoglobulins and being incapable of true adaptive immunity. The validity of distinguishing between the humoral and cellular phases of invertebrate host defences may thus be questionable for certain groups and could obscure important features of invertebrate systems that would further our understanding of immune phylogeny.

Because of their economic importance, size and wide distribution, the Crustacea have been one of the most extensively studied invertebrate groups and a vast literature dealing with nearly every aspect of their immune systems has accumulated. Several comprehensive reviews of the cellular responses in these animals have been produced in the last few years (see e.g. Ratcliffe *et al.*, 1985; Amirante, 1986*a*; Smith & Söderhäll, 1986*a,b*; Johansson & Söderhäll, 1989*a*) but there have been few parallel accounts of humoral immunity or evaluations of the relationship of plasma factors to the cells. Thus, it is timely to reappraise the non-cellular defences of crustaceans in the light of new ideas about non-self recognition and to assess the degree of interaction between cells and plasma. In this overview, humoral immunity is interpreted to include those defence strategies which serve to reduce, neutralise or overcome invasion by non-self material without the direct intervention of intact haemocytes.

2. Humoral Immune Processes in Crustaceans

Humoral defences of invertebrates are usually understood to encompass a multiplicity of serum or plasma factors which act against micro-organisms, foreign cells or abiotic materials. These factors include naturally occurring or inducible bioactive molecules which agglutinate, precipitate or inactivate non-self particles as well as those that have bactericidal, lytic or bacteriostatic properties. Also generally considered as humoral factors are the various fungitoxic, antiviral or cytotoxic agents. Within our definition of non-cellular immunity, we also include clotting because crustaceans, like other arthropods,

characteristically show plasma gelation as well as cell aggregation during haemolymph coagulation (Durliat, 1985). Additionally, we interpret any soluble factors which operate extracellularly and augment the cellular defences (such as cytokine-like molecules, opsonins or cell adhesion molecules) to be part of the non-cellular defence repertoire, irrespective of their site of origin (Table 1).

AGGLUTININS

Factors that bind to and cause the aggregation or agglutination of foreign particles have been reported for many invertebrate species, from sponges to urochordates (see reviews by Schapiro, 1975; Cooper & Lemmi, 1981; Cooper, 1985; Ratcliffe *et al.*, 1985; Amirante, 1986*b*). They have been most extensively investigated in molluscs for which a wide range have been described (see review by Renwrantz, 1986). In crustaceans, agglutinins have been found against vertebrate erythrocytes (see e.g. Cushing, 1967; Cohen, 1968; McKay *et al.*, 1969; Faglioni *et al.*, 1971; Miller *et al.*, 1972; Pauley, 1973; Hall & Rowlands, 1974*a*,*b*; Paterson *et al.*, 1976; Smith & Ratcliffe, 1978; Cornick & Stewart, 1973; Huang *et al.*, 1981; Kamiya *et al.*, 1987; Ravindranath & Paulson, 1987; Ratanapo & Chulavatnatol, 1990), bacteria (Cornick & Stewart, 1968*a*,*b*, 1975; Miller *et al.*, 1972; Huang *et al.*, 1981) invertebrate sperm (Tyler & Scheer, 1945; Smith & Goldstein, 1971), protozoans (Bang, 1962, 1967) and other cells (Tyler & Metz, 1945) (Table 2).

These factors do not occur in every crustacean species and, compared with other invertebrates, the titres are often quite low (Table 2, see also the surveys by Brown *et al.*, 1968; Faglioni *et al.*, 1971). Several agglutinins may be present in any one species but levels of activity may differ considerably between individual animals (Adams, 1991). To date, agglutinins have been found in approximately 40 crustacean species, chiefly decapods (Table 2, see also the surveys by Brown *et al.*, 1968; Faglioni *et al.*, 1971), but there seems to be no clear trend in their occurrence and distribution within the group. In addition, apart from the work of Cohen (1968) and Vasta & Cohen (1984) who reported higher agglutinin titres in larger (older) specimens of the coconut crab, *Birgus latro*, compared to smaller (younger) ones, there has been no proper evaluation of changes in the presence or activity of these factors with age. Similarly, variations in agglutinin titre with respect to the moult cycle are poorly reported although Ghidalia *et al.* (1975) noted no differences in haemagglutinin levels in *Macropipus puber* from moult stage C2 to D2. Furthermore, most studies have been concerned principally with those agglutinins that aggregate vertebrate erythrocytes (haemagglutinins) with fewer investigations made of those that act against bacteria, protozoans or other cells and micro-organisms (Table 2).

For most crustaceans, agglutinins appear to occur naturally in the plasma or serum, although enhanced titres following prior exposure of the host to the test materials have been reported for *Callinectes sapidus* by Pauley (1973) and *Penaeus monodon* by Adams (1991). In both cases, the effect of 'immunisation' is small, short lived and non-specific. There is little evidence that crustacean agglutinins are predominantly inducible and none to suggest that they represent an adaptive element of the host defences or can be exploited for the development of vaccines.

Table 1. Humoral factors or activities in crustaceans or other invertebrates

Factor	Function	Crustaceans	Other Invertebrates
Agglutinins	Aggregate foreign particles. Include bacterial agglutinins, haemagglutinins and/or lectins	Present in nearly all species. Appear to aid sequestration of infective agents but little evidence for a role in recognition	Widely distributed in all groups. Some evidence that haemagglutinins mediate recognition in molluscs and insects
Killing factors:			
Lytic agents	Bactericidal	Bacteriolysins and lysozyme seldom reported. Haemolysins present in spiny lobsters	Bacteriolysins and lysozyme present in most groups, especially molluscs and insects
Peptides	Bactericidal	Not known	Cecropins in insects
Pigments	Microbicidal	Melanin or its precursors are fungicidal and possibly bactericidal	Melanin and its precursors in insects. Echinochrome A in echinoderms
Neutralising factors	Antiviral	Reported for *Callinectes sapidus* and *Carcinus maenas*	Occasional reports but poorly understood
Cytotoxic agents	Destroy cells	Crayfish granular cells are cytotoxic for normal and tumour vertebrate cells *in vitro*	Demonstrated for various phyla. Mechanism unknown

Precipitins	Sequester soluble ' antigens ' from blood	Few reports (mostly in older literature)	Few reports
Cytokines	Non-antibody proteins with diverse immunological and homeostatic functions. Produced by blood cells	Few reports. Factors associated with proPO* activation influence exocytosis, phagocytosis and cell adhesion in decapods	Poorly understood. Some reports for echinoderms
Modulators	Regulate the activities of immunologically aggressive molecules	Poorly studied. a_2 Macroglobulin cages proteinases and is found in crayfish plasma	Poorly studied. a_2 Macroglobulin found in insect and chelicerate blood
Clotting factors	Prevents blood loss and seals wounds	All species. Involves plasma gelation as well as cell aggregation	Cell aggregation in all coelomate groups. Only arthropods display plasma gelation as well as cell aggregation
Recognition factors	Bind specifically to non-self molecules and trigger cell responses	proPO factors released rom cells. β 1,3-glucan-binding factor found in crayfish plasma	proPO factors and glucan-binding factor present in some arthropods. Some evidence for lectin-mediated recognition in insects and molluscs

*proPO = prophenoloxidase activating system.
For references see text.

Table 2. Characteristics of crustacean agglutinins

Species	Test particle	Highest titre	Requirement for divalent cations	Heat stability	Mol.wt. kDa	Source	References
Homarus americanus	rbc*	512	Ca^{2+} for heat	Stable below 56° C	55	Serum	Cornick & Stewart (1968*a*)
	Bacteria	512	Stability at pH 6–9	freeze stable	(subunits)		Cornick & Stewart (1973) Hall & Rowlands (1974*a*) Paterson *et al.* (1976)
Homarus vulgaris	rbc*				200	Haemocyte lysate and whole haemolymph	Durliat & Vranckx (1989)
Panulirus interruptus	rbc*	256 (serum)		Stable below 56° C	Large	Serum	Tyler & Metz (1945)
	Sperm†	256 (serum) 512 (plasma)				Plasma	Tyler & Scheer (1945) Cushing *et al.* (1963)
Cardisoma guanhumi	Sperm†					Whole haemolymph	Smith & Goldstein (1971)
Carcinus maenas	rbc*	64				Plasma	Smith & Ratcliffe (1978)
Callinectes sapidus	rbc*	64	Yes	Stable below 50° C	150	Serum, microsomal membrane	Pauley (1973, 1974) Vasta & Cassels (1983) Cassels *et al.* (1986)
Maia squinado	Protozoa‡					Serum	Bang (1962)
Geryon quinquedens	Bacteria	2				Serum	Cornick & Stewart (1975)
Chionoectes opilio	Bacteria	64				Serum	Cornick & Stewart (1975)
Birgus latro	rbc*	512				Serum	Cohen (1968) Vasta & Cohen (1984)
Cancer irroratus	Bacteria§					Serum	Cornick & Stewart (1968*b*)
Graspus strigatus	rbc*	8				Serum	McKay *et al.* (1969)

Cancer antennarius	rbc*		Ca^{2+} and Mg^{2+}	Stable room temp (5 h) freeze stable	70 (subunit 37)	Serum	Ravindranath *et al.* (1985) Ravindranath & Paulson (1987)
Astacus leptodactylus	rbc*					Haemocyte lysate and whole haemolymph	Durliat & Vranckx (1989)
Parachaeraps bicarinatus (= *Cherax destructor*)	rbc*	128		Inactivated at 57° C		Serum	McKay *et al.* (1969) McKay & Jenkin (1970)
Procambarus clarkii	rbc*	256		Stable below 60° C,	>150	Serum	Miller *et al.* (1972)
	Bacteria	16		inactive at 70° C			
Squilla mantis	rbc*	128	Ca^{2+} and Mg^{2+}	Inactivated at 65° C	193	Plasma	Amirante & Basso (1984)
Macrobrachium rosenbergii	rbc*	256	Ca^{2+}			Serum	Huang *et al.* (1981) Vasta *et al.* (1983)
	Bacteria						
Penaeus monodon	rbc*		Ca^{2+} and Sr^{2+}	Stable below 55° C	420	Whole haemolymph	Adams (1991)
				inactive at 65° C	(subunit 27)	Ovary, testis, muscle and hepatopancreas	Ratanapo & Chulavatnatol (1990)
Triops cancriformis	rbc*			Stable below 56° C		Whole haemolymph	Cenini (1983)
Megabalanus volcano	rbc*	1024	Ca^{2+}	Stable below 60° C	116	Plasma	Kamiya *et al.* (1987)
				inactivated at 80° C	(subunit 23)		

*Vertebrate erythrocytes.
†Invertebrate sperm.
‡*Anophrys sarcophaga*.
§*Gaffkya homari* (= *Aerococcus viridans* var. *homari*).

As far as agglutinins against micro-organisms are concerned, there can be no doubt that these factors must assist in the sequestration of invasive organisms from the haemolymph, thereby facilitating their interaction with the circulating blood cells and preventing the spread of potentially infective agents around the body. It is therefore surprising to find little evidence correlating the occurrence of agglutinins with disease resistance in crustaceans. In an early study, Bang (1962) noted that the spider crab, *Maia squinado* has potent agglutinating activity against the ciliate parasite, *Anophrys sarcophaga*. Unlike the shore crab, *Carcinus maenas* (the normal host of *A. sarcophaga*), *M. squinado* is usually resistant to the protozoan and resistant spider crabs always agglutinate the parasite (Bang, 1962). Interestingly, however, a few individuals of *M. squinado* show susceptibility to *A. sarcophaga* and in these animals agglutinating activity is absent (Bang, 1962). Likewise, with the lobster, *Homarus americanus*, Cornick & Stewart (1968*a*) failed to detect serum agglutinins against the bacterial pathogen, *Gaffkya homari* (= *Aerococcus viridans* var. *homari*), but recorded activity against several other non-pathogenic bacteria. On the other hand, serum from the Atlantic crab, *Cancer irroratus*, for which *G. homari* is mildy pathogenic (Cornick & Stewart, 1975; Newman & Feng, 1982), was found to display weak agglutinating activity against *G. homari* (Cornick & Stewart, 1968*b*) while strong activity was demonstrated in the snow crab, *Chionoecetes opilio*, which is highly resistant to the bacterium (Cornick & Stewart, 1975). Paradoxically, serum from the red crab, *Geryon quinquedens*, fails to agglutinate *G. homari* despite its resistance to infection by this micro-organism (Cornick & Stewart, 1975). Clearly, while antimicrobial agglutinins contribute to protection of the host against disease, resistance does not necessarily reside with any single aspects of the defence network.

The function of the haemagglutinins is less obvious. Typically, they are inhibited by particular sugars and this affinity for polysaccharides has encouraged several workers to consider them as lectins (Table 3). Lectins are defined by the Nomenclature Committee of the International Union of Biochemists as carbohydrate binding proteins of non-immune origin that agglutinate cells or precipitate polysaccharides or glycoconjugates (Liener *et al.*, 1986). They are usually heat-sensitive proteins or glycoproteins with a polyvalent configuration and binding affinity for specific cell surface polysaccharides (Yeaton, 1981*a*). They encompass a huge class of molecules and have been found in plants, micro-organisms and vertebrates as well as invertebrates (Yeaton, 1981*a*,*b*; Liener *et al.*, 1986). Specifically, they do not include carbohydrate binding proteins of known enzymic function, carbohydrate transport proteins or carbohydrate specific antibodies (Goldstein & Hayes, 1978). Partly because of their value as biochemical tools, interest in lectins has increased enormously in the past decade and a wide variety of molecules from a range of sources have been ascribed the status of lectins, albeit inappropriately on occasions. As pointed out by Goldstein *et al.* (1980), an important diagnostic feature of true lectins, which distinguishes them from glycosidases and glycotransferases, is their possession of two or more sugar specific binding sites which enables them to agglutinate foreign cells. Thus, in accordance with the definition cited above, some crustacean haemagglutinins may be reasonably considered as lectins (Table 3). Unfortunately, in other cases, the biochemical character of the

agglutinin has not been properly defined so there may be insufficient grounds for adopting the terminology. This confusion in nomenclature complicates our understanding of haemagglutinin biochemistry and the indiscriminate use of the term lectin may carry unsubstantiated functional implications.

Lectins appear to serve a variety of biological functions (Lis & Sharon, 1986; Liener *et al.*, 1986) and in molluscs, strong evidence has been presented in favour of their role as recognition molecules (see reviews by Ratcliffe *et al.*, 1985; Renwrantz, 1983, 1986). However, information that they serve an equivalent function in crustaceans is either lacking or largely equivocal. With the freshwater crayfish, *Cherax destructor* (formerly *Parachaeraps bicarinatus*), Tyson & Jenkin (1973, 1974) reported significantly enhanced rates of clearance of injected bacteria and *in vitro* phagocytosis following preincubation of the foreign particles in crayfish serum. The serum is known to contain agglutinins against vertebrate erythrocytes and their adsorbtion from the serum was found to abolish its opsonic properties (McKay & Jenkin, 1970). These findings were originally interpreted as evidence for the presence of functional analogues of vertebrate immunoglobulins in invertebrate body fluids (McKay & Jenkin, 1970; Tyson & Jenkin, 1973, 1974). However, when Hall & Rowlands (1974*b*) attempted to show that the haemagglutinins purified from *H. americanus* behave as an opsonin, they obtained only low rates of phagocytosis in their *in vitro* assays despite the presence of the agglutinins. More recently, by fractionisation of lobster haemolymph on Sephadex G-200, Goldenberg & Greenberg (1983) have found the opsonising and agglutinating activities of *H. americanus* haemolymph to be separate and thus it is unlikely that this particular agglutinin acts opsonically. In a previous review of arthropod cellular immunity, Söderhäll & Smith (1986*b*) have discussed the apparent opsonic effect recorded by McKay, Jenkin & Tyson in the light of new findings on prophenoloxidase activation. They suggest that the observed increase in phagocytosis, noted after serum pretreatment of the test particles, could be accounted for by induction of prophenoloxidase activating enzymes in the serum by specific microbial polysaccharides rather than by agglutinin activity. What is needed now is positive identification of the opsonic molecules in crustacean blood and an analysis of their relationship to the other bioactive factors in the haemolymph.

Biochemically, only a few crustacean agglutinins have been characterised. In general, these studies have revealed that most are proteinaceous, heat labile and dependent upon divalent cations, usually calcium ions (Table 2). N-acetylneuraminic acid (sialic acid) specific agglutinins have been reported to occur in the haemolymph of several taxonomically separate crustaceans, including prawns, barnacles and crabs (Table 3). A small number of these have been purified, revealing the presence of multiple agglutinins in several species (Hall & Rowlands, 1974*a*,*b*; Hartman *et al.*, 1978; Amirante & Basso, 1984; Cassels *et al.*, 1986), but data on molecular weights, sedimentation coefficients, amino acid and carbohydrate composition are still scant and sequencing has been carried out only occasionally (Table 3).

The presence of multiple agglutinins in invertebrates has sometimes been taken to support the view that these molecules function in recognition and/or are immunoglobulin precursors. However, the paucity of information about their biochemical structure makes it difficult to assess their biological

Table 3. Characteristics of crustacean lectins

Species	Principal erythrocyte-binding reactions	Sugar specificity	Mol.wt. kDa	Divalent cation requirement	References
Crabs					
Cancer antennarius	Horse, rabbit, rat, mouse	9-*O*-NANA 4-*O*-NANA	70 (dimer)	Ca^{2+} and Mg^{2+}	Ravindranath *et al.* (1985) Ravindranath & Paulson (1987)
Callinectes sapidus	Human A, horse, rabbit, rat	NANA GalNAc GlcNAc ManNAc *N*-acetylmuramic acid *N*-acetylglutamic acid			Cassels *et al.* (1986)
Birgus latro	Human ABO, monkey, birds, rodents	NANA			Cohen (1968) Vasta & Cohen (1984)

Lobster					
Homarus americanus	Human (for LAg1) mouse (for LAg1 and LAg2)	NANA (LAg1) Ga1NAc (LAg2)	55 (subunits)	Ca^{2+}	Cornick & Stewart (1973) Hall & Rowlands (1974*a*,*b*) Hartman *et al.* (1978) Goldenberg & Greenberg (1983)
Shrimps and prawns					
Squilla mantis	Human AO	GalNAc (anti-A) fucose (anti-H)	193	Ca^{2+} and Mg^{2+}	Amirante & Basso (1984)
Penaeus monodon	Human O	NANA	420	Ca^{2+}	Ratanapo & Chulavatnatol (1990)
Macrobrachium rosenbergii	Human ABO, horse, rat, duck, goose	NANA			Vasta *et al.* (1983)
Barnacles					
Megabalanus volcano	Human ABO, rabbit	Lactose Fetuin	116	Ca^{2+}	Kamiya *et al.* (1987)
Megabalanus rosa	Human, rabbit (murine tumour cells)	Galactose	330(BRA1) 140(BRA2) 64(BRA3)	Ca^{2+}	Muramoto *et al.* (1985)

Abbreviations: NANA, *N*-acetylneuraminic acid (=sialic acid); GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine LAg, lobster agglutinin; BRA, *M. rosa* agglutinin.

significance and to evaluate the phylogeny of these molecules across the invertebrates as a whole. A particularly detailed study has recently been made of the structure and amino acid sequence of the agglutinins in the acorn barnacle, *Megabalanus rosa*, by Muramoto and his co-workers (see Muramoto *et al.*, 1985; Muramoto & Kamiya, 1986, 1990*a*,*b*). Here, up to three galactose-binding agglutinins have been identified; each with a different molecular weight, designated BRA-1 (M_r330 000), BRA-2 (M_r140 000) and BRA-3 (M_r64 000). Both BRA-1 and BRA-2 are composed of identical subunits (M_r22 000) which are cross linked by interchain disulphide bonds while BRA-3 is composed of four identical subunits (M_r16 000) of 138 amino acids. Analysis of their amino acid sequences has revealed that they show marked homology to other invertebrate agglutinins as well as to C-type (calcium dependant) lectins of vertebrates, but not to plant lectins or vertebrate S-type agglutinins. This suggests that vertebrate and invertebrate agglutinins might have a common ancestral gene but until more invertebrate agglutinins are examined, the phylogeny of these molecules remains speculative (Muramoto & Kamiya, 1990*a*). Interestingly, although the presence of highly conserved cysteine residues (involved in the disulphide bridges) in the barnacle agglutinin is reminiscent of the heavy chain segments of human immunoglobulins, no similarity exists with immunoglobulin variable regions (Muramoto & Kamiya, 1986, 1990*b*).

Perhaps because of the desire to find a link between the recognition molecules of vertebrates and invertebrates, there has been an assumption by nearly all investigators that agglutinins form a major part of the humoral, as opposed to the cellular, defences in invertebrates. However, the origin of these factors in most crustacean species has not been established and in a few instances they appear to be associated with the cell membranes (Vasta & Cassels, 1983; Cassels *et al.*, 1986). Moreover, in many studies it is serum (i.e. the fluid remaining after clotting) rather than plasma, which has been tested for agglutinating activity. Crustacean haemocytes are remarkable for their extreme sensitivity to endotoxin and other non-self materials and rapidly undergo exocytosis or lysis *in vitro* (Smith & Söderhäll, 1983*a*; Söderhäll *et al.*, 1986) (see below). The accompanying activation of previously cell bound enzymes by the foreign molecules releases a number of 'sticky' proteins (Söderhäll *et al.*, 1983) which could bring about the extracellular aggregation of the non-self particles *in vitro*. Even where plasma fractions have been examined, adequate precautions against haemocyte lysis during sample preparation have not always been taken, making it difficult to determine the extent to which any agglutinating effect could be cell-derived. Overall, the site(s) of agglutinin synthesis in crustaceans remains obscure. In some cases they have been found in association with the cells but in other instances they have not. For example, Cornick & Stewart (1973) have noted that agglutinin activity is associated with the haemocytes in *H. americanus*, and Amirante & Basso (1984) have demonstrated, with monoclonal antibodies, that the haemagglutinin in *S. mantis* is present on the membranes of the granular haemocytes as well as free in the plasma. More recently, Ratanapo & Chulavatnatol (1990) have reported that the agglutinin in *P. monodon* occurs in the ovary, testis, hepatopancreas and muscle as well as the haemolymph. By contrast, Smith & Söderhäll (1983*b*) have found that in *C. maenas*, while weak haemagglutinating activity toward sheep, horse and

human erythrocytes resides in the serum, none is present in lysate supernatants of the haemocytes. The absence of haemagglutinating activity in these extracts does not necessarily preclude the location of agglutinins on the haemocyte surface but shows that in this species they are not present in a soluble form within the cells.

KILLING FACTORS

A wide variety of substances that kill foreign cells or micro-organisms have been described for invertebrates (see reviews by Cooper & Lemmi, 1981; Cooper, 1985; Ratcliffe *et al.*, 1985). They are ubiquitous throughout the Invertebrata and may act against bacteria, fungi, viruses, protozoans or other foreign cells. In general, these factors are non-specific and comprise a heterogeneous collection of molecules. Their killing effects range from general disinfection, as with the pigment, echinochrome A from echinoderms (Service & Wardlaw, 1985), to lysis of defined peptidoglycan bonds, as is the case for lysozyme. Some, such as the didemnins from tropical sea squirts (Rinehart *et al.*, 1981, 1983) or the cecropins and attacins from lepidopteran insects (Boman, 1986, 1991), represent unique families of antimicrobial molecules and may have potential therapeutic value as antibacterial, antiviral or antineoplastic agents.

In crustaceans, killing factors have been reported relatively infrequently and they have tended to receive rather less scrutiny than the agglutinins. They encompass factors that are effective against viruses (McCumber *et al.*, 1979) bacteria (Evans *et al.*, 1968, 1969*a,b*; Weinheimer *et al.*, 1969*a*; Stewart & Zwicker, 1972; Mori & Stewart, 1978*a*; Söderhäll & Smith, 1986*a*; Adams, 1991; Chisholm & Smith, 1991) or fungi (Nyhlén & Unestam, 1980) (Table 4) but ones against protozoans or other parasitic animals have seldom been described. Haemolysins have also been reported in a few species, for example, in *M. squinado* (Cantacuzène, 1920), *P. argus* (Weinheimer *et al.*, 1969*b*) and *Triops cancriformis* (Cenini, 1983). However, little is known about these factors or the extent to which they contribute to antimicrobial defence.

Bactericidins have attracted most attention, especially in crabs and lobsters (Table 4), probably because of the high value of these animals in aquaculture. Typically, bactericidal activity in lobsters appears to be effective against a variety of bacteria, the main exception being the pathogen, *G. homari* (Cornick & Stewart, 1968*a,b*, 1975; Stewart & Zwicker, 1972) (Table 4). The lack of killing towards *G. homari* undoubtedly contributes to its pathogenicity as the haemolymph provides an excellent nutritive medium for growth (Cornick & Stewart, 1968*a*). Bactericidins against *G. homari* are also absent from the haemolymph of the crabs (Cornick & Stewart, 1968*b*, 1975; Chisholm & Smith, in prep.) which are either resistant or only mildly susceptible to the pathogen (Cornick & Stewart, 1975; Newman & Feng, 1982).

Otherwise, studies have been concerned primarily with the host response to Gram-negative bacteria, including isolates from the gut of the host animal, rather than Gram-positive forms (Evans *et al.*, 1968, 1969*a*; Weinheimer *et al.*, 1969*a*; Mori & Stewart, 1978*a*). Recently, we have undertaken a detailed survey of bactericidal activity by *C. maenas* using haemocyte lysate supernatants (HLS) to challenge 12 different strains of marine bacteria. We have found that,

Table 4. Antibacterial, antiviral and fungitoxic factors in crustacean haemolymph

Species	Test organisms	Inducibility	Requirement for divalent cations	Heat stability	Comments	References
Panulirus argus	Gram-negative bacteria	Yes	No	Stable below 60° C	Not agglutination	Evans *et al.* (1968)
				Inactive at 65° C	Enhanced 2° and 3° responses	Evans *et al.* (1969*a*)
				Freeze stable −25° C		Weinheimer *et al.* (1969*a*)
Panulirus interruptus	Gram-negative bacteria	Yes		Stable at 60° C	Not agglutination	Evans *et al.* (1969*b*)
				Inactive at 65° C	2° responses noted	
Homarus americanus	*Pseudomonas perolens* and *Aerococcus viridans* var. *homari*	Yes			Mainly in hepatopancreas	Acton *et al.* (1969) Stewart & Zwicker (1972) Mori & Stewart (1978*a*,*b*)
Callinectes sapidus	Bacteriophages Poliovirus		No	Stable at 56° C	Plasma more effective than serum	McCumber *et al.* (1979)
					6-13S 80 kDa polymer	McCumber & Clem (1983)
Carcinus maenas	Marine bacteria		No	Stable at 60° C	Maximally effective against Gram-negative organisms active at high titre	Söderhäll & Smith (1986*a*) Chisholm & Smith (1991)
				Freeze stable at −20° C and −70° C		
Penaeus monodon	Gram-negative bacteria	Yes			Partial specificity to vibrios	Adams (1991)
Pascifastacus leniusculus	*Aphanomyces astaci*				May be due to melanin or melanin precursors	Nyhlén & Unestam (1980)

whereas killing is strongest towards Gram-negative organisms, Gram-positive organisms are also affected, including strains isolated from geographically remote areas (Chisholm & Smith, 1991). As yet we do not know whether killing in this animal is brought about by a single broad spectrum factor or through a battery of separate bactericidal agents.

In many cases, killing activity has been found to be induced by pretreatment of the host with live or heat-killed bacteria (Evans *et al.*, 1968, 1969*a*,*b*; Acton *et al.*, 1969; Weinheimer *et al.*, 1969*a*; Stewart & Zwicker, 1972; Mori & Stewart, 1978*b*; Adams, 1991) (Table 4). However, these responses appear to have limited specificity and the time taken to reach maximum effect is highly variable, ranging from as little as 36–48 h (Evans *et al.*, 1968, 1969*a*; Adams, 1991) to 7 days (Evans *et al.*, 1969*b*). So far the phenomenon has not been properly explained. Analyses of the protein composition of the plasma after stimulation have not been carried out and no work has been done to determine whether pre-exposure of the host provokes protein synthesis by the tissues, as is the case with lepidopteran insects (see review by Boman, 1986). It is well-established that injection of foreign agents into the haemocoele of crustaceans produces a marked haemocytopaenia within a few hours, and recovery of the cell number can take at least 2 days (Smith & Ratcliffe, 1980; Smith *et al.*, 1984). The extent to which changes in the number of circulating cells (and quantities of bioactive substances released by them) are related to the induction of bactericidins has not been assessed. It would therefore be relevant to discover whether, after foreign onslaught, the internal stimulus to mobilize cells and/or accelerate cell division in the haemopoietic tissue simultaneously triggers synthesis of antimicrobial agents within the host body.

Certainly, as with the agglutinins, killing does not appear to be restricted to the serum or plasma. While microbicidins occur in the haemolymph of some species (Stewart & Zwicker, 1972; Mori & Stewart, 1978*a*; Adams, 1991), in others, they may be associated with different tissues (Mori & Stewart, 1978*b*). In lobsters, bactericidal activity has been found to reside predominantly in the hepatopancreas and the small amount present in the haemolymph is not associated with the haemocytes (Mori & Stewart, 1978*b*). By contrast, although Smith & Ratcliffe (1978) and White *et al.* (1985) have failed to detect bactericidal or bacteriostatic factors in the plasma or serum of *C. maenas*, Söderhäll & Smith (1986*a*) and Chisholm & Smith (1991) have noted strong activity in haemocyte lysate supernatants (see above). More recently, Chisholm (unpubl.) has obtained some preliminary results which indicate that, under certain conditions, cell-free haemolymph from *C. maenas* displays bactericidal properties. As yet the reason for these conflicting results with shore crabs has not been resolved but a possible explanation might lie with the procedure used to prepare the plasma. In *H. americanus*, Stewart & Zwicker (1972) have reported that bactericidal activity exists *in vivo* largely in an inactive form and requires co-operation with a factor within the haemocytes for full expression. If the techniques used for plasma preparation cause leakage of the cells, the resulting sample could be contaminated with cell-derived material, thus affecting killing ability.

Biochemically, little is known about the killing factors in crustaceans. Most have no requirement for divalent cations, which distinguishes them from the

agglutinins (see above), and the majority are inactivated above 60° C but are stable upon freezing to −20° C (Evans *et al.*, 1968; Chisholm & Smith, in prep.) (Table 4). In other respects, biochemical characterisation is incomplete and the dearth of information hinders our ability to give a satisfactory account of the mechanisms underlying bacterial killing. Furthermore, the extent to which bactericidal activity is discrete from bacteriostasis has not always been made clear and it is uncertain whether the foreign agents are destroyed by lysis or other means.

Lysozyme, an enzyme capable of splitting the β-1,4 glycosidic links of bacterial cell walls, has not been reported for crustaceans, although it exists in many other invertebrate groups, especially insects and molluscs (see review by Ratcliffe *et al.*, 1985). Likewise, there is virtually no evidence for a peroxidase-mediated antimicrobial pathway in crustaceans (White *et al.*, 1985; Hose *et al.*, 1987), despite the importance of this system in antibacterial defence in vertebrates (Klebanoff, 1975).

Recently, Boman (1991) has discussed the role of peptides in antibacterial defence and pointed out that in terms of their broad specificity, lack of reactivity against eucaryotic cells, rapid diffusion rates and relative ease of synthesis, they have unique advantages in non-specific host defence. More importantly, from a phylogenetic standpoint, there is no need for synthesis by specialised cells and they do not require a repertoire of recognition molecules for effect. They are thus ideal candidates for primitive defence molecules. Such bacteriolytic peptides are known to occur in lepidopteran insects, in the form of the cecropins (see review by Boman, 1986). These molecules are induced by bacterial challenge and are highly potent towards both Gram-negative and Gram-positive bacteria. All have 31–39 residues, are devoid of cysteine and have a strongly basic N-terminal region and a long hydrophobic stretch in the C-terminal half (Boman, 1991). The occurrence of these factors is not restricted to lepidopterans as similar molecules have now been detected in other insects and also in pig intestine, showing that they have been conserved during evolution and are widely distributed within the animal kingdom (see Boman, 1991). As yet, cecropins have not been identified in crustaceans, but it is not unreasonable to predict that they might be found, bearing in mind the close phylogenetic relationship of these animals with the insects. The possibility that peptides could be responsible for some of the antibacterial activities reported in crustacean tissues should not be dismissed and investigation of the presence and structure of these molecules is a potentially exciting area for future research.

Whether peptides play a role in the bacterial killing observed in *C. maenas*, however, remains enigmatic. So far, the phenomenon appears to be achieved without lysis, as there is no reduction in bacterial turbidity following incubation of bacterial suspensions with haemocyte extracts (Chisholm & Smith, in prep.). In addition, clearing has not been observed on bacterial lawns challenged with samples known to contain high titres of the bactericidal factor (Chisholm & Smith, in prep.). Since the killing factor in *C. maenas* resides principally in the granular cells (Söderhäll & Smith, 1986*a*), it is possible that factors associated with the activation of prophenoloxidase, contained within these cells, may be involved. Previously, Unestam (1975) and Nyhlén & Unestam (1980) have observed the deposition of melanin around fungal hyphae in the tissues of the

crayfish. Söderhäll & Ajaxon (1982) have subsequently demonstrated that quinones and melanin, the products of phenoloxidase activation, are fungitoxic, thereby giving credence to the suggestion of Nyhlén & Unestam (1980) that melanin or its precursors behave as inhibitors of fungal enzymes. Interestingly, Söderhäll *et al.* (1985) have shown that crayfish, *Astacus astacus*, haemocytes display potent cytotoxicity towards ^{51}Cr-labelled human or mouse tumour cells *in vitro*, but that cell lysates, containing an active prophenoloxidase activating system, have no lytic properties. Probably, a variety of killing strategies operate within the crustacean group, but it would be pertinent to determine the exact role of the prophenoloxidase system in extracellular killing and to ascertain whether components of the system have other antimicrobial properties.

With respect to antiviral activity, Taylor *et al.* (1964) were the first to demonstrate that crustaceans are able to clear viruses from the haemolymph. Later, McCumber & Clem (1977) showed that clearance is accompanied by the deposition of these agents in the tissues, although the final destination of the sequestered virus is variable. T_2 and T_4 bacteriophage, for example, are cleared to the hepatopancreas whereas poliovirus is deposited in the gills (McCumber & Clem, 1983). The response seems to be augmented by a neutralising factor in the haemolymph, which is unrelated to either haemocyanin or the haemagglutinin for mouse erythrocytes (McCumber *et al.*, 1979). Significantly, plasma is more effective than the serum in neutralising bacteriophage (McCumber *et al.*, 1979) and preliminary characterisation has established that in *C. sapidus*, the factor responsible is a polymer (6–13 S) of non-covalently linked subunits, each with a molecular weight of *c.* 80 kDa (McCumber *et al.*, 1979). However, our understanding of anti-viral defence in crustaceans is in general rather poor and work to support and extend these preliminary findings is urgently required.

PRECIPITINS AND OTHER FACTORS

Precipitins have been reported only occasionally for invertebrates, and in the Crustacea their presence is noted only in the older literature. Interest in these factors was probably connected with early ambitions to discover antibody-like substances in lower animals but, in parallel with our changing view of non-self recognition processes in invertebrates, enquiry into precipitins has declined. One of the first descriptions for crustaceans was by Osawa & Yambuui (1963) who found weak precipitin activity in *Cambarus clarkii*, following inoculation with sheep erythrocytes. Later, Stewart & Foley (1969) recorded a naturally occurring precipitin to fluorescein-labelled bovine serum albumin in *H. americanus*. In this latter case, the factor responsible was found to be dialysable and stable to 50° C, but to be present at low titres (Stewart & Foley, 1969). Furthermore, as there was no accumulation of label within the circulating cells, there did not appear to be any direct cellular intervention in the removal of this protein from the blood (Stewart & Foley, 1969).

More recently, in an elegant series of investigations, Clem and his co-workers have shown that in *C. sapidus*, radioactively labelled proteins injected into the haemolymph are very rapidly cleared from the circulation in a dose-independant manner which does not rely on factors present in the plasma (McCumber & Clem, 1977; Clem *et al.*, 1984). Curiously, the response does not seem to involve

intervention of the circulating cells as there is no binding of the proteins to the haemocytes and extirpation of 99% of the haemocytes fails to impair protein clearance (Clem *et al.*, 1984). Furthermore, immunisation has no stimulatory effect on the rate of clearance of secondary challenge; instead, exposure of the experimental animals to relatively large doses of the protein immediately prior to injection of the labelled proteins tends to slow down their removal from the blood (Clem *et al.*, 1984). Because of the absence of plasma factors and lack of haemocytic involvement, the mechanism for the elimination of non-self proteins in the animal is unclear. Significantly, the proteins become localised in the gills (McCumber & Clem, 1977) and fixed cells (such as the nephrocytes) in these structures might play some role in their removal from the blood (Clem *et al.*, 1984). Evidence that a fixed reticuloendothelial-type network exists in crustaceans has been presented by Smith & Ratcliffe (1981) who demonstrated that whilst the nephrocytes in *C. maenas* are not actively involved in the sequestration of injected bacteria from the blood, they accumulate debris generated during the formation of haemocyte clumps (nodules) *in vivo*. Confirmation of the role of the nephrocytes in protein elimination awaits identification of the appropriate receptors on their cell surface, but, once again, it seems that, in crustaceans, there might be a cellular aspect to an erstwhile humoral immune response. Clearly, the presence or absence of precipitins in crustacean plasma needs to be fully evaluated as does the existence of protein-binding molecules on the surface of the nephrocytes.

Aside from precipitins, another factor that has been found in crustacean plasma is a glucan-binding molecule (Duvic & Söderhäll, 1991). This monomeric glycoprotein has a molecular mass of 100 kDa and binds to laminarin (Duvic & Söderhäll, 1991), a specific elicitor of the prophenoloxidase activating system (Söderhäll & Unestam, 1979). The factor enhances the activation of haemocyte-derived peptidase and prophenoloxidase by laminarin, but has no peptidase or phenoloxidase properties of its own and is not an agglutinin or lectin (Duvic & Söderhäll, 1991). In amino acid composition and biological activity, this glucan binding factor resembles an equivalent protein in insect plasma (Ochiai & Ashida, 1988; Söderhäll *et al.*, 1988) and is thought to assist in recognition by triggering the phenoloxidase cascade, not through phenoloxidase directly, but through the activating serine potease (Duvic & Söderhäll, 1991). Interestingly, the protein also seems to be located in the circulating haemocytes (Duvic & Söderhäll, 1991) but whether it is secreted by these cells is, at present, unknown.

CYTOKINE-LIKE FACTORS

Cytokines (or, as they are more usually known in connection with vertebrate immune systems, lymphokines) are non-antibody proteins, produced, in mammals, by activated lymphocytes. They assist in homeostatic regulation of the immune system and facilitate integration of the immune response with other body processes, by, for instance, controlling the production and maturation of the different cell types, influencing neuro-endocrine function, mediating acute phase responsiveness, affecting cell movement or displaying cytotoxic properties of their own (Adams, 1982).

It is debatable whether equivalent factors can be said to exist in invertebrates, mainly because of the constraint their definition imposes, being strongly biased towards vertebrates. In terms of blood cell-derived factors that mediate, promote or modify immune reactivity, there are a few cases where cytokine-like activity can be reasonably claimed for lower animals, principally in starfish (Leclerc *et al.*, 1981; Prendergast *et al.*, 1983; see also review by Ratcliffe *et al.*, 1985). As regards crustaceans, there is, unfortunately, very little information available. There are a number of factors produced by activated haemocytes that have a controlled influence on the host defences, usually in connection with the induction of the prophenoloxidase system, but whether these may be designated cytokines is largely a matter of opinion. Certainly, the granular haemocytes of decapods release proteins (opsonins) that significantly step up the rate of phagocytosis by the hyaline cells (Smith & Söderhäll, 1983*b*; Söderhäll *et al.*, 1986) and generate a factor that stimulates cell degranulation (Johansson & Söderhäll, 1989*b*). Moreover, Johansson & Söderhäll (1988) and Kobayashi *et al.* (1990) have isolated and purified a 76 kDa protein from crayfish granular cells that promotes cell adhesion to foreign surfaces as well as to other cells during encapsulation. However, chemotactic agents or substances that stimulate haemopoiesis or direct neuro-endocrine function have not been found. That such molecules might occur in crustaceans is plausible, especially as activation of the prophenoloxidase system is known to involve proteolytic cleavages which generate small peptides (see review by Söderhäll & Smith, 1986*a*) and, in vertebrates, these types of molecules are known to play key roles in various aspects of homeostasis.

MODULATORS

With respect to immune modulation, there has been little attempt to determine how the agglutinating, lytic or biocidal agents in crustacean blood are regulated, despite the obvious need for such molecules to prevent inappropriate or massive intravascular triggering of the defence system. One factor that has been found in crayfish plasma is a high molecular weight proteinase inhibitor (Hergenhahn & Söderhäll, 1985; Stocker *et al.*, 1991) which resembles a_2-macroglobulin (a_2-M) of vertebrates (Hergenhahn *et al.*, 1988; Hall *et al.*, 1989). It is known that in vertebrates, a_2-M has the effect of 'caging' proteinases, thereby reducing their activities towards large substrates but leaving intact their proteolytic effect on small substrates (Sottrup-Jensen *et al.*, 1989). The crayfish factor, like that of mammalian a_2-M, inhibits the activity of trypsin and chymotrypsin and contains a basic monomeric M_r polypeptide subunit of high molecular weight (*c.* 155 kDa in *P. leniusculus*) (Hergenhahn *et al.*, 1987). This high M_r inhibitor further blocks the serine protease associated with prophenoloxidase activation and is believed to serve in a regulatory capacity for the enzyme cascade (Hergenhahn *et al.*, 1987). In crustaceans, a_2-M-like activity has been described mainly for the plasma, but in the horseshoe crabs, has also been found to be associated with the circulating amoebocytes (Armstrong *et al.*, 1990). At the moment, we do not know if this also obtains for decapods or is a situation unique to chelicerates because of their particular form of clotting (see below).

CLOTTING

Clotting is an essential process in all coelomate animals which serves to seal wounds and stem the loss of blood upon injury. It is now well-established that clotting in most invertebrate groups entails aggregation of the circulating cells, but in arthropods the process also involves the gelation of the plasma by factors released from the haemocytes (Grégoire, 1971). The phenomenon has been most extensively studied in the chelicerates, mainly *Limulus polyphemus* and its close relatives, and several excellent accounts of clotting in arthropods have already been published (Durliat, 1985; Levin, 1985; Bohn 1986; see also review by Ratcliffe *et al.*, 1985). It would be inappropriate to attempt a further review here, so instead, important points in relation to the present article are briefly discussed.

Firstly, in most crustaceans, clotting is mediated through coagulogens present in the plasma and also compartmentalised within the circulating cells. The plasma factor has some similarity to fibrinogen of vertebrates and is converted to covalently linked polymers of coagulin by Ca^{2+}-dependent transglutaminase (see Durliat, 1985). By contrast, the cell factor is converted to a gel by a serine protease pro-clotting enzyme which may be triggered by lipopolysaccharide (LPS) or β1,3-glucans (Durliat, 1985; Durliat & Vranckx, 1989). In this way, it is linked to the prophenoloxidase activating system (Söderhäll, 1981, 1982; Söderhäll & Smith, 1986*a*) and is probably discharged from the cells by LPS or other non-self stimuli (Smith & Söderhäll, 1983*a*; Johansson & Söderhäll, 1985) whence it reacts with the plasma factor to form the clot (Durliat, 1985; Durliat & Vranckx, 1989). Similar events occur in insects, and in these invertebrates, the plasma and cell coagulogens have no immunological identity and are highly partitioned, i.e. there is no evidence of plasma coagulogen in the haemocytes (Barwig & Bohn, 1980; Bohn *et al.*, 1981; Bohn, 1986). More importantly, in macruran crustaceans, the plasma factor cannot gel without haemocyte clumping whereas haemocyte lysate supernatants containing the cellular coagulogen are able to undergo a clotting reaction independantly of the plasma, providing protease activity has been induced by the appropriate non-self molecules (Söderhäll, 1981; Durliat, 1985; Durliat & Vranckx, 1989). Thus, clotting furnishes a good example of the way in which the blood cells and plasma factors interact to bring about an effective response by the host. The only exception for this pattern in the Crustacea seems to be the parasite, *Sacculina carcini*, which has a clear, acellular, non-circulating coagulable liquid, presumably plasma, in the space between the basilar membrane and mantle (Levin, 1967). This fluid forms a gel and is capable of immobilising planktonic ciliates without cellular intervention (Levin, 1967). Bacteria or bacterially-derived endotoxins also stimulate gelation of the plasma (Levin, 1967), although exhaustion of blood coagulability by these agents usually results in the development of bacteraemia and death of the sacculine, often with the subsequent demise of its decapod host (Levin, 1967).

Conversely, in the Limulidae, all the factors required for coagulation of the blood seem to be located inside the circulating amoebocytes (Levin, 1985). The main clotting protein comprises a single polypeptide chain which is converted to a non-covalently cross-linked gel through a serine protease (Durliat, 1985;

Levin, 1985). This protease is similar to the one present in the prophenoloxidase cascade of other arthropods, and may be activated via two pathways; one stimulated by endotoxin (LPS) the other by β 1,3-glucans (Kakinuma *et al.*, 1981; Morita *et al.*, 1981). Other similarities exist between the clotting pathway of chelicerates and the prophenoloxidase activating system of crustaceans but the main difference is the absence of the terminal component, phenoloxidase, from the *Limulus* system (Söderhäll & Smith, 1986*a*).

This link between the clotting system and the phenoloxidase cascade in arthropods has important implications for the phylogeny of immunity, as discussed previously by Söderhäll & Smith (1986*a*) and Smith (1991). In particular, the exocytosis of previously cell bound coagulogen provides a mechanism for the simultaneous release of other bioactive molecules. Some of these, especially those associated with prophenoloxidase activation, have been shown to enhance or induce haemocyte responsiveness, as is the case with the opsonins, cell adhesion and cell degranulation factors described above. These factors are produced by one cell type (usually the granular or semigranular cells) but may exert their influence on a different type (Söderhäll *et al.*, 1986). In this way, clotting may generate signals that mediate cell to cell communication in crustacean immunity and concomitantly, other cell-derived factors might be released during this process that aggregate, kill or neutralise invasive microorganisms. Accordingly, it is not unreasonable to predict that some of the factors mentioned in the preceding sections could be associated with clotting through the prophenoloxidase system.

3. Effect of Environment

Of increasing concern, particularly in relation to the success of aquaculture ventures, is the effect of environment (e.g. season, temperature, diet, reproduction and pollution stress) on immunity, and hence disease resistance, by the host. However, for most crustaceans, virtually nothing is known about the impact of environmental parameters on the defence networks.

A few authors have recorded variations in humoral activity in response to changes in temperature or season. For example, Dean & Vernberg (1966) have found that temperature affects clotting times, total haemocyte count and levels of plasma protein in the hermit crab, *Uca pugilator*. More recently, Muramoto *et al.* (1991) have recorded higher agglutinin activity in the acorn barnacle *Megabalanus rosa* during the summer months compared with the winter, although it is unclear whether this is a direct consequence of temperature or is due to other physiological factors. Certainly, temperature is a major determinant of the metabolic and cellular processes and has an all pervasive influence on the biology of poikilothermic animals, but the extent to which it influences immunocompetence in crustaceans is largely unknown. With respect to microbial killing, Karen Bell in our laboratory has recently found that acclimation temperature has a powerful effect on *in vitro* bactericidal activity of *C. maenas* (unpubl. res.). Here, killing of *Planococcus citreus* or a marine Pseudomonad, designated strain 1-1-1, is lower in animals acclimated to 19° C or 13° C compared to control animals maintained at 6° C (Fig. 1). Associated with

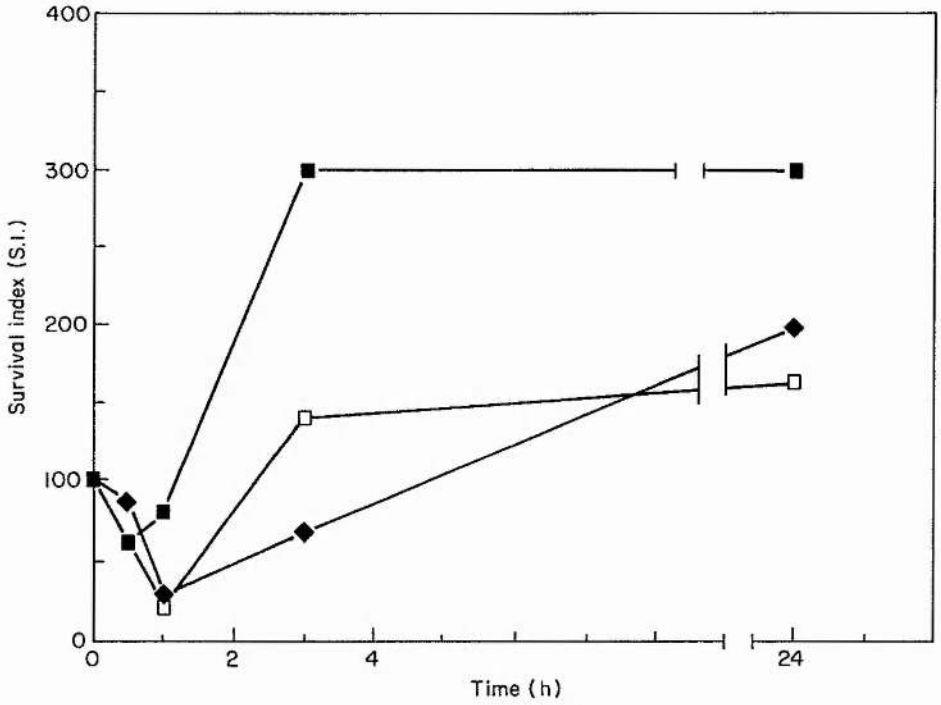

Fig. 1. Effect of acclimation temperature on *in vitro* killing of the Gram-positive marine bacterium, *Planococcus citreus* (NCMB 1493), by crab, *Carcinus maenas*, haemocyte lysate supernatants (HLS) (Bell, unpubl.). The survival index represents the percentage survival of the test bacteria in the experimental mixtures at each time interval compared to controls. Crab HLS was prepared as described in Smith & Söderhäll (1983*b*). Controls consisted of *P. citreus* incubated in saline supplemented with 0·5% peptone broth instead of HLS. (—□—), 6° C; (—◆—), 13° C; (—■—), 19° C.

this is a concomitant reduction in the haemocyte count and the level of blood cell phenoloxidase (Table 5) (Bell, unpubl.). This change in haemocyte number may not account for the effect of temperature on the killing response in crabs entirely since related *in vitro* experiments with non-acclimated crabs have revealed better killing in experimental mixtures incubated at 20° C than those incubated at 0° C (Chisholm & Smith, in prep.). These findings suggest that in crustaceans a reduction in immune capability is part of the physiological response to thermal stress; a matter of considerable importance in relation to global warming.

With pollution, despite the almost universal use of crustacean species, such as *Daphnia* and *Crangon*, as experimental animals in traditional LC_{50} toxicity tests, virtually nothing is known about the effects of environmental stress, particularly chemical pollutants, on the immune systems of crustaceans. In other invertebrates, there have been a few analyses of xenobiotics on the humoral defences. For example, in insects, Jones *et al.* (1989) have demonstrated that exposure of the cabbage white butterfly, *Pieris brassicae*, to 20 ppm 2,4,5-trichlorophenoxyacetic acid significantly lowers haemolymph bacterial activity and reduces levels of serum lysozyme. Likewise, in molluscs, exposure of the clam, *Mercenaria mercenaria*, to heavy metals has been shown to cause destabilisation of the haemocyte lysomal membrane with the consequent release of acid phosphatase into the haemolymph (Suresh & Mohandas, 1990).

Table 5. Effect of acclimation temperature on total haemocyte count and phenoloxidase activity in the crab, *Carcinus maenas* (Bell, unpubl.)

	Acclimation temperature			
	6° C*	13° C	19° C	*N*§
Haemocyte count ($ml^{-1} \times 10^8$)†	22·5 ± 10·8‡	6·0 ± 2·7	5·35 ± 6·0	5
Phenoloxidase activity (units)‖	1·8	1·35	0·323	5

*Ambient seawater temperature at the time of the experiment was 6° C. Experimental animals were gradually acclimated to the higher temperatures for 2 weeks prior to bleeding.

†Haemocyte counts were made on individual animals using Improved Neubauer haemocytometers.

‡Values given are means ± S.D.

§*N* = No. of animals tested.

‖Phenoloxidase activity in haemocyte lysate supernatants was estimated spectrophotometrically at 490 nm using L-dopa as substrate and trypsin as elicitor as in Smith & Söderhäll (1983*b*). Enzyme activities are expressed as units where one unit represents the change in absorbance per minute per mg protein.

Continued exposure to pollutants inhibits its activity and blocks further synthesis of the enzyme by the blood cells (Suresh & Mohandas, 1990).

In crustaceans, Smith & Johnston (1991) have recently found that short-term exposure of the common shrimp, *Crangon crangon*, to sub-acute concentrations of polychlorinated biphenyls (PCBs) *in vitro* results in depression of phenoloxidase activity in haemocytes and suppression of the total blood cell count. More importantly, this study also revealed that there is a differential haemal response according to the PCB congener used and in a manner that could not be predicted from immunological studies of vertebrates (Johnston *et al.*, 1991; Smith & Johnston, 1991). As yet, the implications of this PCB induced haemotoxicity in disease resistance have not been tested experimentally, but it would appear that as elements of the recognition and effector branches of the immune system are affected, resistance to microbial attack might be impaired.

4. Conclusions

Crustaceans possess a wide range of non-cellular factors that complement the responses of the circulating cells in the protection of the host against disease or non-self agents. Of these humoral factors, most investigations have been directed at agglutinins, although a repertoire of microbicidal, lytic and precipitating molecules have also been reported. Much still needs to be learnt about these factors, particularly with respect to their biochemical nature and origin. Likewise, the way these molecules are regulated/modulated *in vivo* needs to be ascertained, as does the existence of cytokines or cytokine-like proteins. In addition, greater consideration should be given to the role of the non-cellular agents in defence against viruses, protozoans and helminth parasites, both in normal and in environmentally stressed animals.

One important and consistent feature to emerge from the present evaluation is the confusion pertaining to the relationship of the humoral factors to the haemocytes and other tissues. In a small number of cases, the humoral factors appear to reside exclusively in the plasma and to operate independently of the

circulating cells. In many other instances, however, they are closely allied with cellular activity and may be derived from the cells during infection or foreign challenge. Certainly, it would seem that non-self recognition and host defence in crustaceans are achieved only by the combined effects of the haemocytes with substances present in the plasma. This interdependence of the cellular and humoral branches of the defence system has important implications. Firstly, the development of biphasic immune reactivity permits localization of the defence response to the site(s) of infection without incurring the large scale liberation of immunologically aggressive molecules into the general circulation. Secondly, it facilitates the operation of a feedback mechanism to regulate and control the activities of these molecules, thus preventing the detrimental effects of inappropriate triggering of the defence system *in vivo*. Thirdly, it enables cell signalling pathways to function. Some of these biochemical signals appear to be linked to the activation of prophenoloxidase and, if, as research is beginning to indicate, this system is an elaboration of the clotting pathway, then the exocytosis of clotting factors from the haemocytes could have provided the framework for the evolution of the cell messenger systems in these animals.

Another key point is the paucity of evidence for inducibility of the humoral defences in crustaceans. Apart from killing factors, primary exposure to foreignness has been shown to promote subsequent secondary challenge of 'antigens' in only a few cases and frequently there is no satisfactory explanation for the observed phenomenon. This has bearing on attempts to develop vaccines for commercially valuable species. Without doubt, the absence of clonally derived specific antibody type molecules in crustaceans means that the production of vertebrate style vaccines is unrealistic. Whether it will be possible to stimulate the synthesis of other broad spectrum antimicrobial agents for prolonged periods is still unproven. To date, there is only sparse evidence that immunisation confers protection on the host through the induction of microbicidins in the blood. Enhanced secondary and tertiary responses have been noted in only two species of spiny lobster (Evans *et al.*, 1969*b*; Weinheimer *et al.*, 1969*a*) and in neither case was the effect large. As discussed above, the injection of non-self materials adversely affects the number of circulating haemocytes in crustaceans which could abrogate any apparent beneficial effects that immunization might have. In order to promote immunocompetence in crustaceans, further research into the synthesis and mobilisation of antimicrobial factors, whatever their source, is necessary.

Finally, the effect of environmental factors on humoral immunity in crustaceans is very poorly understood. There is presently much concern about the immunosuppressive influence of certain pollutants on marine or freshwater invertebrates and interest is being generated in the identification of novel biomarkers for sub-acute toxic effects. The potential of the non-cellular defences as indicators of environmental perturbation is still unexplored despite the obvious advantages that blood parameters offer in terms of non-sacrificial accessibility, sensitivity and known physiological importance.

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